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SEX STUDIES

XI. HERMAPHRODITE BIRDS¹

ALICE M. BORING AND RAYMOND PEARL

NINE TEXT FIGURES AND NINE PLATES

I. INTRODUCTION

Studies IX and X of this series have dealt with the normal structure of the reproductive organs in the domestic chicken. That matter was undertaken as a preliminary to the study of certain hermaphrodite birds which have come into the possession of the Maine Agricultural Experiment Station. It is only possible to interpret abnormal conditions in the light of and by comparison with the normal. The present study undertakes a description of the external sex characters and internal reproductive structures of these abnormal birds. The general bearing of both normal and abnormal structures in the reproductive systems of the chicken on theories of secondary sex characters will be discussed in still another paper, which has to be delayed for some time as one of the authors (R. P.) has been called by the government to turn his attention to war time problems.

II. MATERIAL AND METHODS

The material for this study comprises a large number of birds belonging to the poultry plant of the Maine Agricultural experiment Station. The work has centered around eight hermaphrodite birds. Five of these birds were bought from Herr Houwink, a well known writer on problems of heredity and evolution in poultry, in Meppel, Holland. They are Drentisch fowls, the common breed of North Holland. He states that "these fowls

¹ Papers from the Biological Laboratory of the Maine Agricultural Experiment Station No. 116.

are cultivated by inbreeding." In 1909, he started this particular stock with one cock and six hens. From then on, he has inbred them. In the F_2 generation in 1911 there appeared two hermaphrodites out of 80 birds, and in F_3 in 1912, there were three more hermaphrodites out of 80 birds. These are the five birds sent to Maine. These were by no means all the birds of the sort which Herr Houwink had produced. When one of the writers (R. P.) visited his breeding plant in Meppel in 1910 there were then on hand a considerable number of these supposed hermaphrodite birds. Herr Houwink was of the opinion, as already stated, that inbreeding was the cause of these abnormalities, but without producing evidence which was conclusive to this effect.

These Holland birds were numbered 1425, 1426, 1427, 1428 and 1429 on the Experiment Station poultry plant, and will go by these numbers in this paper. One of the other three hermaphrodites was a small black bird sent to the Experiment Station by Prof. Horace Atwood of West Virginia. This will be referred to as Atwood's black hermaphrodite. The seventh hermaphrodite appeared in the Station flock of pure Barred Plymouth Rock ancestry. The number of this bird is 1349. The eighth bird, 1616, was sent from Michigan by Professor Dexter. These eight birds vary in the proportion of male and female in their make-up, both as to external and internal structure, and behavior. It seems clearest to arrange them in a series from the most female at one end to the most male at the other end as the various features of structure and behavior are taken up, using the structure of the primary sex organs as the basis for this serial arrangement.

This study has been carried a little further and included other birds with abnormal sex behavior, but normal structure. That is, to complete the more female end of the series, we have studied several apparently normal hens that tread other hens. Of these, 1422 was sent from West Virginia by Professor Atwood as a possible hermaphrodite but has behaved normally in Maine. K134 and M408 were two such birds reared at the Station plant and finally killed to study their anatomy and histology.

To complete the more male end of the series we have studied two guinea-chicken hybrids, one raised on the Maine poultry plant and the other sent in for examination from the country. Their gonads appeared to be testes, but their behavior was indifferent.

This series of birds has been studied from as many aspects as possible. The external secondary sex characters have been observed and many of the birds photographed to show any deviations from the normal. The behavior of the entire series has been carefully watched under various conditions and recorded. The birds were finally killed to study the anatomy and histology of the reproductive organs. The alimentary tract and associated organs were removed and a piece of the back with the entire urogenital system was put in McClendon's fluid (10 per cent formalin, sugar, lime) to fix and harden. Then a careful dissection was made of the urogenital system, and this was photographed. The reproductive organs and ducts were then sectioned and studied histologically. Delafield's haematoxylin and orange G were used for the general histological condition of the glands. The more special staining methods for studying interstitial cells have been considered in detail in study IX of this series.

III. EXTERNAL SEX CHARACTERS AND SEX BEHAVIOR

Secondary sexual characters are very distinct and attain a relatively high degree of development in the domestic fowl. The cock bird is distinguished from the hen in a whole series of obvious respects. Of these the best known and most apparent are; (1) greater body size; (2) different shape of body and carriage; (3) large comb and wattles; (4) presence of spurs; (5) presence of differentiated feathers in hackle, saddle and tail, (6) in some breeds dimorphism in color and pattern. There are many finer differences which can be recognized by the poultry expert.

Presence of testes is supposed to cause the appearance of one set of these characters; presence of ovary, the appearance of the other. Actually in some ten years study of these characters in

poultry at the Maine Station it has been found that there is no single one of the so-called male secondary sex characters which has not at some time been seen well developed in an otherwise perfectly normal female. Laying females with spurs, or with large, male-like comb and wattles, while not common, still appear every year in small numbers amongst the large numbers of birds in the whole flock. Females with male secondary plumage are rarer but still occur. Putting all our experience together we have come finally to the view that there is no distinct secondary sexual character for which the correlation with the primary organ is perfect and unvarying.

Taking a general survey of the external characters of the birds in this study, and taking especially into account the shape of body and body carriage, they would fall into line from most female to most male in the same order as mentioned above,—that is, the sum total of external characters, and particularly the body shape and carriage, would correspond roughly to the internal anatomy. They cannot be compared as to body size as they were not all of the same breed nor were they of the same age when killed for anatomical study.

Various female birds in the Station flock, which are normal laying birds at times tread the other hens. Two such birds, K134 and M408 were studied for comparison with the abnormal birds. These were entirely normal females as to all external structural characters. K134 was a Barred Plymouth Rock and Black Hamburg cross. She laid 184 eggs in 1913-14, and 84 from October to June of 1914-15, at which time she was killed. M408 was a Rhode Island Red and had laid 96 eggs up to the time of her death which occurred before she was a year old. No. 1422 was a White Leghorn sent to the Station by Professor Atwood of West Virginia, because he thought she might be a hermaphrodite. Her behavior, however, at Maine was that of a perfectly normal female. Her egg record shows 25 eggs during the two months of September and October while she was at Maine. During the winter she laid no more and died in February. There were no male second sex characters present in any of these birds. We shall see later in detail that this habit of

treading hens has no evident basis in the structure of the reproductive apparatus.

The external characters of four of the Holland birds is shown in their photographs (figs. 1 to 4). Unfortunately 1425 died before she was photographed. None of these birds look like entirely normal females or males. No. 1429 (fig. 1) appears the most female and 1426 (fig. 4) the most male. They all have large combs, 1426 the largest, and 1428 the smallest. The wattles on 1426 and 1428 are larger than on the other two. The spurs are large on 1427 and 1429, and very small on 1426 and 1428. Quite evidently these three so-called secondary sex characters do not correspond with body shape and carriage, as the most male has small spurs and the most female large comb and spurs. None of these five birds ever laid an egg or showed any sex behavior. They stood around the pens in a perfectly indifferent manner and never offered to fight either pullets or cockerels.

Atwood's hermaphrodite was also unfortunately not photographed. The records show that she had a small comb, large spurs, male carriage, female body shape, and that she was henny-feathered. She had been presented to the Station by Mr. Atwood of West Virginia because of her hermaphrodite characteristics. Her behavior was watched while she was here at the poultry plant, and that proved to be also hermaphrodite in that the bird fought both males and females. Normally in the fowl females fight only with females and males only with males. She is recorded as "a great fighter." In fact, she met her death as a result of a fight after she had been transferred from one pen to another. The detailed account of structure in a later section of this paper shows that this bird had a hermaphrodite gonad, but that the germ cells were immature.

Bird 1349 was raised on the Station poultry plant, a cross between a Barred Plymouth Rock and a Game. It was clearly a female when it first reached maturity, but gradually developed more and more male characters, until at the time of its death, it was rather strongly male of the game type. Figure 5 was photographed during the moult, but shows the male carriage. The size of comb and spurs was typically male at the time of

death. There were no wattles, the head being typically female in this regard. There were no true sickle feathers in the tail, although the tail was better developed, i.e., more towards the male type than in the normal female. There were no typical male hackle or saddle feathers. This bird was completely a hermaphrodite as to behavior, as it was observed to act alternately as a male and as a female in copulation. It never laid an egg. The bird was generally kept in a coop by itself except when being watched under special conditions to record its behavior. When put in a pen with pullets, it would act as a male, but as a female when put in with the cockerels. Finally one day when it was put down in a yard with some other hens to observe its behavior, it started to fight and was dead in five minutes. Reference in the later sections of this paper will show that the structure of the gonad at the time of death indicated that it was changing over from ovary to testis.

Bird 1616 (fig. 6) was sent to the Station as an abnormal cockerel by Dr. Dexter from Michigan in May, 1915. The bird was a cross between a Rhode Island Red male and a Plymouth Rock female. He was a year old when sent to Maine. For the Michigan period of his life, he is described as entirely hen-feathered and except for the head which is that of a rooster, he looked like a hen. He was very active and crowed a great deal more than usual. The other roosters chased him all over the farm, and the hens would not permit him to copulate with them, although he made frequent attempts to do so. After he reached Maine the female characters became more pronounced and the bird was recorded as a crowing hen. On July 31 she laid an egg, and between that time and August 25 she laid 12 eggs in all and nested twice. From then on she laid no more eggs and began to become more malelike in appearance. She was killed on March 15, 1916, and at that time her external characters were a mixture of male and female. She had no spurs, merely slight knobs such as all females have. She was very fat. She had no saddle feathers, but the hackle feathers were partly male. These hermaphrodite external characters correspond to the anatomical conditions described later—that is, the bird has an ovotestis, with

indications of recent or present activity in both the male and female parts of the gonad.

The study of the external characters and behavior of these hermaphrodite birds shows a great variety of combinations. Evidently the body shape and carriage and the plumage hang together more consistently as secondary sex characters than the spurs, comb and wattles. The latter group vary too much to be considered as proofs of maleness or femaleness. Sex behavior varies all the way from complete indifference to active reproduction. Three of the birds show double sex behavior acting as a male or female under different conditions at the same general period, or showing a gradual change from the behavior of one sex to that of the other. A case somewhat similar to that of 1616 has been described by O. N. Eastman in the *Poultry Advocate* for September, 1916. At first he did not know whether to class this bird as a pullet or a cockerel, but as she became more mature she looked like a pullet with a head like a cockerel. She began to lay in November, 1915, while housed in a pen with pullets and one cockerel. This one cockerel chased her so incessantly, as one male bird does another, that she had to be removed to a pen with only pullets. In August, 1916, she was seen to chase and mate with a pullet, and she repeated this behavior several times. This comparison will be taken up again after the full description of the anatomy and histology of the sex organs of these birds.

The two guinea chicken hybrids were entirely male in external characters, but absolutely indifferent as to behavior. They stood around the pens in much the same way as the Holland birds, taking no interest in either males or females. We shall see later this indifferent behavior is not accompanied by any gross abnormalities in the form of the reproductive organs—that is, not to such anomalies as an oviduct or oocytes, but to a lack of differentiation in the testis tissue.

IV. WOLFFIAN DUCTS IN NORMAL FEMALE BIRDS

Before describing the internal structures of this series of abnormal birds, it is necessary to mention certain points of normal bird anatomy which have been observed in this connection. Many of these points have already been described by Goodale. Our own observations are recorded here simply in corroboration of his, and because in certain particulars our evidence is more detailed. The normal reproductive system of a male bird comprises two testes and two vasa deferentia. The normal reproductive system of a female bird includes a left ovary and a left oviduct. The right ovary and oviduct start to develop in the embryo, but stop before long so that they do not function in the adult. By the fifth or six day, according to Semon, the right ovary is already smaller than the left. The right oviduct forms as a right Mullerian duct, and usually degenerates along with the ovary. However, this duct may sometimes continue to grow and persist in the adult as a non-functioning oviduct. There are fairly frequent cases of this recorded among the autopsies of the birds of the Maine Experiment Station poultry plant.

Every embryo female chick has besides its two gonads and two Mullerian ducts, two Wolffian ducts which have been supposed to degenerate in the female as the Mullerian ducts do in the male. The statement in Lillie's "Development of the Chick" reads as follows: "In embryos that become females, the gonad develops into an ovary, the Wolffian duct disappears or becomes rudimentary, the Mullerian duct develops into the oviduct on the left side and disappears on the right side."

In studying the anatomy of the hermaphrodite birds, the kind of ducts present was at first taken as an indication of sex—that is, the presence of vasa deferentia was regarded as a sign of maleness, and the presence of an oviduct as the corresponding sign of femaleness. According to this criterion, all these birds were hermaphroditic as they had a left oviduct and two vasa. The vasa were small ducts which had to be searched for in the peritoneum but sections showed them to be tubes lined with columnar

epithelium, surrounding a distinct lumen, and therefore impossible to be confused with either bloodvessels or nerves.

The same condition of ducts was found in bird No. 1422, the bird sent from West Virginia with a record of treading hens. Everything else about the anatomy of this bird was that of a normal female—she laid eggs and showed no abnormal behavior after reaching Maine. The suspicion arose that these small non-functional vasa deferentia of the hermaphrodites might signify simply an embryonic condition,—that is, persisting Wolffian ducts—rather than maleness. Further, the fact of finding them present in 1422, otherwise a normal female, suggested the possibility of their being a normal feature of the anatomy of an adult female bird. In Lillie's "Development of the Chick" there occurs the following statement: "In the female, the Wolffian duct degenerates; at what time is not stated in the literature, but presumably along with the Wolffian body." The persistence of the ducts of the other sex in adult vertebrates is not an unheard of phenomenon,—in fact, it is the normal condition in the common leopard frog for the Mullerian ducts to persist in the adult male.

To work out this point, as to how long the Wolffian ducts persist in the female bird, dissections were made of a number of just hatched chicks and chicks from pipped eggs, seven of which proved to be females, and of five laying hens. All of them had Wolffian ducts. They were not as large as in the male—in fact, sometimes they looked like white threads along the peritoneum lateral to the ureter at the posterior end, crossing it about half way between cloaca and gonad, and extending further anterior than the ureter near the midline to the remnants of the mesonephros. Sometimes they were as large as normal, but never had as many coils at the posterior end. Figure 7 is a dissection of one of the laying hens. To be sure that this white line was not a nerve or bloodvessel, parts of it were sectioned in each bird. The columnar epithelial lining identified it unmistakably (figs. 8 and 9). Figure 8 shows the vas alongside of an artery, a vein, and the ureter. Each is easily identified. These ducts show variation in structure. Sometimes

they are single straight tubes, sometimes they show the characteristic coils of a vas. Always toward the anterior end, and sometimes posteriorly also they have branches. These are probably remnants of the connection with the mesonephric tubules in the embryo.

In the laying hens, it is sometimes hard to identify the Wolffian duct on the left side on account of the coils of the large oviduct. That it persists on the left as well as on the right side, however, is established beyond a doubt by the fact that it was found on some of the laying hens, and also by the fact that all seven of the young female chicks had two Wolffian ducts. We agree then with Goodale that the presence of Wolffian ducts in an adult bird is not necessarily a sign of maleness.

V. ANATOMY AND HISTOLOGY OF ABNORMAL BIRDS

The internal structure of this series of abnormal birds shows varying degrees of abnormality, and the interest of the study lies chiefly in seeing whether there is a close correspondence between these and the abnormalities of external structure and behavior. We have seen that the normal female has two Wolffian ducts of varying sizes, besides the left ovary and oviduct, so that the presence of Wolffian ducts is not a sign of maleness. No oviduct has, however, been found in any male, so its presence may be considered a sign of femaleness of internal structure. The external appearance of the reproductive organs has proved to be insufficient to distinguish between an ovary and a testis. An organ with a few projecting oocytes may be partly testis, and an organ without any visible oocytes may be ovary, testis or both. In deciding whether certain tissue is ovary or testis, the only indisputable criterion is when it has oocytes or spermatozoa. The general structure of the organ is, however, usually sufficient to show the difference in sex even when in an inactive condition, the testis being composed of tubules with a small quantity of connective tissue between them, and the ovary being largely stroma. However, there are some intermediate conditions found when it is difficult to sex the organ as they both develop from a stage when the sex cords grow

out from the mesonephros into the germinal epithelium. The interstitial cells might be used as an index of sex, because of their consistent absence in Barred Plymouth Rock males over six months of age, if it were not for the fact that the gonads in some of these hermaphrodites seem to be cases of arrested development, and interstitial cells have been found in young just hatched males. The nests or groups of clear cells which normally fill up the discharged follicles and form the 'corpus luteum' are never found in the normal male. So their presence may be counted for femaleness.

Many cases of hermaphrodite birds have been described by various authors, with varying degrees of maleness and femaleness combined. More of them seem to be females which have developed some male characters than vice versa. Some of these abnormal combinations of external characters are associated with corresponding internal abnormalities and some are not. We shall discuss these cases after we have described the anatomical and histological conditions found in the hermaphrodites with which the present study is concerned.

In the description of their anatomy and histology the birds will be taken up in the order stated above—that is, beginning at the more female end of the series. Three of the birds with normal external characters and abnormal behavior were killed and dissected. No. 1432 was an entirely normal female with ovary and oviduct on the left, and persistent Wolffian ducts. K134 and M408 were killed and dissected and portions of the ovary preserved and sectioned. Both of these also proved to be normal females in structure. The ovaries were large with many protruding oocytes of varying ages. The sections showed also many small oocytes embedded in the surface layer of the ovary. Both luteal cells and interstitial cells were present, in the same general arrangement and number as in the normal laying hen. It would seem then that the abnormal maleness of behavior in these three birds does not depend on any abnormalities of structure, there being no male organs present, or any male cells in the ovary, and there is present the full quota of female organs and cells.

We shall consider next the Holland birds, and begin with the most female of them. No. 1429 is shown in dissection in figure 10. This is a photograph of a part of the back of the bird with all viscera removed except the urogenital system. The posterior end can be distinguished by the small piece of rectum remaining where the digestive tract was cut off slightly above the cloaca. The ducts can be seen, all connecting with the cloaca, and extending anteriorly to the region of the reproductive organ which lies at the most anterior end of the dissection. This one reproductive organ lies to the left and is an ovary with many oocytes visible to the naked eye, and a few very small orange spots like remnants of corpora lutea. The large round dark object to the left of the ovary is a tumor more than twice the size of the ovary. There is a normal oviduct with coils but the bird never laid an egg while in the Station flock. The two median ducts are the ureters. The right Wolffian duct shows its entire length plainly from cloaca to a spot opposite the ovary. It is somewhat coiled at the posterior end. At the anterior end, there is a slight enlargement, which proves in section to be a mass of tubules, resembling an epididymis. This is probably the remains of the mesonephric tubules, sometimes spoken of as a parovarium. A small portion of the left Wolffian duct shows in the photograph median to the oviduct. This is also somewhat coiled. There seems to be nothing male about the anatomy of this bird. It has an ovary and oviduct on the left and two Wolffian ducts. The only abnormal feature is the large tumor, which, of course, shows a diseased condition. The anatomy of the bird is, in short, that of a female in the non-laying condition with a large tumor. The histological study of the ovary shows it to differ in some points from both the old Campine past the laying condition and the actively laying birds, described in studies IX and X. In general it resembles the Campine more closely; that is, there is a large relative amount of stroma and there are no very small developing oocytes (fig. 18). The oocytes present are of medium size and lie in normal follicles. In addition there are a number of cystic follicles filled with a watery fluid. These are visible to

the naked eye, and some show in section in figure 18. There is the usual large number of nests of luteal cells in the theca interna, as shown in figure A. Some of these luteal cells present a feature not seen in any other bird, they contain a large number of acidophile granules. That these cells with granules are not the interstitial cells can be seen by comparing figures A and B. Figure B is a group of interstitial cells from the stroma of this same ovary. The interstitial cells are smaller and more closely packed with granules. The only difference among the cells in figure A is that some are clear and some have granules. They are the same as to size and nucleus. The number of real interstitial cells is small (fig. C). In various parts of the gonad, there are older luteal cells with the yellow pigment. Whether these corpora lutea represent discharged or atretic follicles it is impossible to decide, as the involution process has proceeded beyond the stage where this distinction can be made. The microscopical structure together with the gross anatomy show 1439 to be a female.

There is not much choice as to the order in which the next three birds shall be described, as the ducts of all three are of the female type, and the reproductive organs of all are indifferent enough to make it somewhat difficult to sex them.

The dissection of 1428 is shown in figure 11. The two Wolfian ducts are easily seen in this photograph, being of considerable size. The bird has a larger coiled oviduct than 1429, and a lobulated reproductive organ on the left. This organ has a large watery tumor to the left of it, showing that it is also in an abnormal physiological condition. It is exceedingly difficult to sex this organ. It is largely composed of tubules, which radiate toward the periphery from a central connective tissue core. But the entire surface looks like an ovarian stroma (fig. 19) and all this connective tissue at the periphery and continuing down between the tubules contains many masses of the luteal cells normally found in the theca interna (figs. 19 *t* and 20 *t*). The tubules are in some places lined with characteristic columnar epithelium cells (fig. 20), but in most the cells appear to be breaking down (fig. 19). In the central core of

this organ, there are some interstitial cells loaded with secretion granules (fig. D) also many streaks of tumor-like material. This bird is probably a female arrested in the development of its gonad earlier than 1429. To be sure



Fig. A Portion of follicle wall from ovary of 1429 ($\times 570$). *G*, epithelial layer; *N*, nest of luteal cells in theca interna; *A*, luteal cells containing acidophile granules.

Fig. B Portion of stroma of ovary from 1429 showing a few interstitial cells loaded with granules ($\times 570$).

Fig. C Portion of periphery of ovary from 1429, showing the small number of interstitial cells present ($\times 264$).

the tubules look like testicular structures, but they may instead be mesonephric, a condition which might be found in an embryonic ovary. In fact, it looks much like the ovary of the just-hatched chick shown in figure 32. The presence of the luteal cells in groups in the stroma also suggest arrested development, as in the normal development of the egg follicle, there is a stage before the theca layers are added when only the granulosa is present, but the nests of luteal cells are conspicuous in the stroma near the young follicles. Although the luteal cells are present they have not gone through their full development,



Fig. D Portion of connective tissue core of gonad from 1428, showing numerous interstitial cells ($\times 264$).

as there is no trace of the yellow pigment in any part of the gonad. The dissection of 1427 is shown in figure 12. The two Wolffian ducts are shown in the photograph, the right one with a distinctly enlarged anterior end, which is of the nature of a mass of mesonephric tubules or parovarium, as shown by section. This bird has also a coiled oviduct and a lobulated reproductive organ on the left. Here again is a large dark tumor posterior to the reproductive organ as in 1429. Figure 21 is a section of this organ. It seems largely composed of solid cords of cells. There are no oocytes and no hollow tubes,

so it is difficult to be sure of the sex. A comparison with sections of just-hatched ovaries and testes seems to throw a little light on its nature. Even at this stage the testis has distinct tubules, but in the ovary, the oocytes are not yet enclosed in follicles, but the germinal epithelium has grown down into the stroma in solid cords (fig. 32). The appearance of the cortex is not unlike that of 1427. Sections of 1427 stained with Mallory's connective tissue stain show no interstitial cells present, and also no luteal cells. The probable conclusion then as to the internal structure of 1427 is that it is a female with an inactive gonad even less differentiated than in 1428. Development was probably checked by some pathological condition, of which the large tumor may be an index.

The dissection of 1425 (fig. 13) is not very different from that of 1428 or 1427 just described, except for the absence of a visible tumor. Sections show the posterior portion of the gonad to be filled with streaks of a secretion which resembles the substance of the tumor in 1429. This would indicate that it is in a similar pathological condition, although no separate tumor has been formed. Externally the reproductive organs of these three birds could scarcely be distinguished. Internally, however, the gonad of 1425 is more like that of 1428, in that the central portion is composed of tubules with distinct lumina, as shown in figure 22. There is no sign of any mitoses in any of the tubule cells, so they are probably of mesonephric origin,—that is, undifferentiated sex cords without any primitive germ cells. The peripheral portion has probably as in 1428 originated from the germinal epithelium. There are no interstitial cells present in the stroma, and the number of groups of luteal cells between the tubules is less than in 1428. The presence of any of the latter clinches the diagnosis of this bird as a female arrested in development. The fewness of these cells may place it as more primitive than 1428,—that is, in between 1428 and 1427. Unfortunately there is no record or photograph of external characters to compare with the other two.

The last of the Holland birds in the series, 1426, is distinctly different from the others in its structure. It has two repro-

ductive organs, and the large coiled oviduct of a laying bird (fig. 14). At the posterior end, to the right of the cloaca is a crumpled mass, which is apparently a partly developed right oviduct. No Wolffian duct was found on the left side, but the immense size of the oviduct made it difficult to dissect on that side. The organs of the two sides are very different in external appearance, as can easily be seen in the photograph. The right one is an active testis and the left one an inactive ovary. Figure 23 is a photograph of a section of the testis, a large mass of tubules with very little connective tissue between them. Part of this same section is shown at greater magnification in figure 24. This resembles a section of a normal active testis. The black threads are fully formed spermatozoa, and they are bunched into groups for each Sertoli cell in normal manner. The vas, strange to say, is no larger than in the birds with no organ on this side, and not as much coiled as the one in 1429. The left reproductive organ closely resembles the ones in 1428, 1425 and 1427 in external appearance. On the surface are a couple of depressions which might be degenerate oocytes or discharged follicles. One place shows an orange mass like a corpus luteum. Histologically the main substance is like that of 1427, and resembles the stroma of an ovary with solid cords of cells in it, but no oocytes (fig. 25). The peripheral tissue, however, looks like a thickened germinal epithelium and contains several large spaces which may have been oocytes. In the place with the orange spot on the surface there is a mass of tissue containing groups of cells with yellow pigment material, as described for discharged or atretic follicles in study X. The entire stroma shows great streaklike masses of secretion taking acid stains, as in the organs of 1425 and 1428. In one limited area of the ovary, there are a few interstitial cells filled with granules. In both ovary and testis there are a few nests of luteal cells near the surface. This condition in the testis is shown in figure E, as it is unusual to find them in a testis, and probably indicates the generally unbalanced sex condition of the animal which looks as though it might be changing from female to male. This is the most interesting of the Holland birds, absolutely indifferent as to

sex behavior and yet with sperm in the testis, and at least one corpus luteum remnant on the ovary, and the oviduct of a laying hen. In external appearance, it is more like a male than the others, which fact correlates well with the active condition of the testis and inactive diseased ovary with only one corpus luteum scar. The interstitial cells can scarcely be held accountable for the male secondary sex characters, as the only ones in

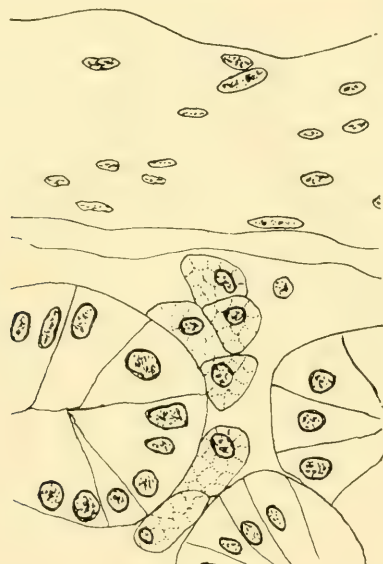


Fig. E Portion of testis from 1426, showing some luteal cells between the seminal tubules at the periphery ($\times 570$).

an active secreting condition are a few in the ovary. This bird is the most balanced hermaphrodite of the series, and closely resembles bird 16, described by Pearl and Curtis, as to its internal structure, except for the active condition of the testis.

A comparison of these five Holland birds shows a general correspondence between the degree of femaleness of their external characters and their internal structure. They are all fundamentally females in which the ovary for some reason has failed to reach complete development. In all but 1429 and 1426 the luteal cells are immature. In 1426, a testis has developed

on the right side. They are all of them, however, entirely indifferent as to sex behavior, and such so-called secondary sex characters as comb, wattles, and spurs vary regardless of internal structure. The interstitial cells vary too much in their distribution to be considered the cause of the general maleness or femaleness of the external characters, and they cannot have anything to do with the large combs and spurs, as the distribution of the two does not correspond. For example, 1427 has no interstitial cells and a very well developed comb and spurs. No. 1428 with the most interstitial cells has the smallest spurs. No. 1429 in spite of having the most female carriage and most normal ovary has longer spurs than 1426, which is the most male of the series. Spurs and combs are too variable to be considered distinctively male secondary sex characters. On the other hand, the distribution of the interstitial cells does not correspond with either the maleness or femaleness of body shape and carriage. However, the only one of these five birds with any luteal pigment worth mentioning is 1429, the one at the more female end of the series. This may be significant. The cause back of the lack of development of oocytes and luteal secretion may be the general abnormal physiological conditions indicated by the tumors and pus present.

We shall consider next the anatomy and histology of the three birds with hermaphrodite behavior. Atwood's black hermaphrodite has two Wolffian ducts and an infantile oviduct (fig. 15). On the left is a large irregular organ. The left half of this lefthand reproductive organ is not unlike the organs found in 1427, 1425 and 1428,—that is, it is irregularly lobed, but the one largest posterior lobe looks more like a testis in the smoothness of its surface than any of the other organs referred to. The right half of the left hand organ appears like an ovary with small oocytes all over the surface and two small orange spots like corpus luteum remains. Sections show this organ to be an ovotestis. The part which appears externally like a testis is composed of tubules. In none of them are any advanced stages of spermatogenesis; the majority of the cells

are spermatogonial cells or spermatocytes in synizesis. The portion that resembles an ovary in external appearance has distinct ovarian tissue on the periphery, but the center is filled with tubules in an even less developed condition than those of the part already described (fig. 26), but distinctly testicular as they are filled solid with cells, not hollow like mesonephric tubules. On the right side of the body there is an enlargement of the anterior end of the Wolffian duct and this, upon being sectioned, appears like the center of the ovarian portion, a mass of small tubules with inactive cells (fig. 27). The ovarian portion of this organ contains oocytes of many sizes. Some of them are contained in normal follicles with the characteristic nests of luteal cells in the theca interna, but the majority are beginning to degenerate. Many groups of these luteal cells lie in the connective tissue of the stroma between the follicles and a few among the testis tubules at the center of the ovary and among the tubules of the small testis on the right. In three places the sections passed through atretic follicles packed with these cells here containing clumps of the yellow luteal pigment. This histological structure represents the orange spots visible on the surface to the naked eye. No interstitial cells were found in any portion of the organ. This bird is a potential hermaphrodite in its internal structure, a fact of especial interest in view of its hermaphroditic behavior. The structure looks as though it were changing from female to male. The oocytes are mostly starting to degenerate and some atretic follicles are already filled with luteal cells containing the characteristic pigment. The presence of luteal cells among the testis tubules looks as though the tubules were growing from the center outward and forcing their way into the ovarian tissue at the surface. But neither the ovarian or testicular tissue was in active condition when the bird was killed.

No. 1349 is the second bird with hermaphrodite behavior. Figure 16 is a dissection of the reproductive organs. In this bird, they were dissected out of the body and preserved as photographed before the Wolffian duct situation had been worked out, so that it is possible that the bird possessed the normal

two ducts. The organs shown in the photograph are a coiled oviduct and a gland which proves to be mostly a testis (fig. 28). Some of the tubules show spermatids and developing sperm (fig. 29). This organ was sectioned in eleven different regions and only three showed any structures other than testis tubules. One of these parts is shown in figure 30. This strongly resembles the indifferent ovary of 1426 and 1427, so we are probably justified in calling this an ovotestis. One other portion which was not testicular is most remarkable in structure (fig. F). It is a large solid collection of smaller masses of luteal cells partly degenerated and containing a few yellow pigment granules (fig. G). There is enough of this pigment to give the mass a yellowish tinge to the naked eye. There are also nests of luteal cells in normal undegenerated condition between the tubules of the testicular portion, as shown in figure H. This organ looks as though it had been an ovary and was largely changed over to a testis. It was certainly mostly testis when the bird was killed. But the yellow pigment must represent either discharged or atretic egg follicles and the groups of luteal cells between the tubules suggest that these tubules have somewhat recently invaded ovarian tissue. It is especially significant that these groups of luteal cells lie mostly toward the surface of the gonad.

These two birds with active sex behavior have reproductive organs in a less active condition than 1426, with absolutely indifferent behavior. In fact, 1426 has mature sperm in the testes, while 1349 has either immature or degenerate sperm, and Atwood's bird had cells no further developed than the synizesis stage. On the female side, the oviduct of 1426 is much larger and more coiled than that of either of the other two birds, but they all have signs of having had active ovaries,—that is, they have several degenerating oocytes, many immature luteal cells, and several luteal pigment masses. No. 1426 has less of the ovarian tissue remnants, just as it has a more advanced testicular structure. There are a few interstitial cells in 1426, but none in either of the active birds, so that active sex behavior can scarcely be based on these. Also the differences cannot be laid to the luteal cells, as they are present in all three.



Fig. F Section of old ovarian tissue from gonad of 1349, showing stroma filled with groups of luteal cells resembling those in discharged or atretic follicles ($\times 100$).

Fig. G Group of luteal cells from figure F, showing masses of yellow secretions ($\times 950$).

The third of these genuine hermaphrodites is 1616, the bird sent by Dr. Dexter from Michigan. The anatomy of its reproductive organs is shown in figure 17. There is a large coiled oviduct and two Wolffian ducts. There is one large lobulated reproductive organ on the left. This is larger than in any of the other birds studied. The surface of this organ has oocytes of varying sizes scattered at various places, but the whole texture of the lobes is more solid than in a normal ovary. There are fourteen corpora lutea remnants. The egg record of this bird shows that she laid 12 eggs and nested twice, so that in this case, the orange spots just represent discharged instead



Fig. H Section of testicular tissue from gonad of 1349, showing a group of luteal cells in a space between seminal tubules ($\times 570$).

of atretic follicles. At the anterior end of the right Wolffian duct is the same sort of enlargement as has been mentioned for Atwood's bird. The microscopical study of the large organ shows it to be an active ovotestis with the testis portion in the more active condition when the bird was killed. The main body of the organ is testis tubules with sperm in the lumens. The small organ on the right is also active testis with sperm. But the peripheral portion of the large organ is distinctly ovarian. It consists of thickened stroma packed with interstitial cells like the old Campine (fig. I). It contains oocytes of all sizes, small ones with only a granulosa layer to the follicle and large ones with nests of luteal cells in the theca interna. There

are not nearly as many oocytes as in a normal ovary. Finally it contains a few discharged follicles; one recently discharged with the cavity still large and the *granulosa* sloughing off into it, a second with the cavity just obliterated by the shrinking of the walls, and a number with the degenerating luteal cells in the center containing the yellow pigment, exactly as in normal birds. This is normal ovarian tissue, but there is not so much of it as there is of the testicular tissue. The composition of this organ is more like Atwood's bird than any other, in the proportion of male and female parts. The point in which this bird

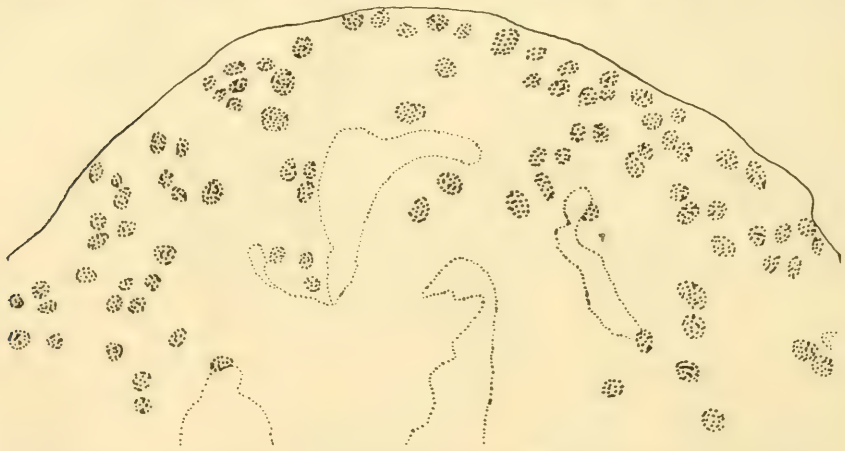


Fig. I Portion of periphery of gonad from 1616, showing numerous interstitial cells ($\times 264$).

differs from all others studied is that both the ovary and the testis show signs of very recent or present activity.

It is interesting to compare the structures found in these birds with those of some of the hermaphrodite birds previously described by other authors. In 1889, Brandt described eight hermaphrodites of varying structure, seven of which he considers modified females. In 1906, Shattock and Seligmann described a two year old Leghorn with an ovotestis. Also of interest are the hermaphrodites with ovary and testis described by Pearl and Curtis, the four mule pheasants, which are sterile females with some male secondary sex characters, described by

Smith and Thomas, and a gynondromorph pheasant described by Bond in 1914. The oviduct is an almost constant feature in these birds. The only bird without an oviduct is one described in Brandt's paper. Shattock and Seligmann's Leghorn had two oviducts. Five of Brandt's birds had abnormal oviducts, either anterior or posterior end being closed. In the rest, the female ducts were normal, just as in the birds described in this paper. In most of these papers, nothing is said about the vasa deferentia. Brandt shows one picture of sections through two persistent Wolffian ducts, the one on the right side being larger than the one on the left. In the bird which he claims is a male with female characters developed, he says there are no vasa deferentia. Pearl and Curtis's bird 16 had an oviduct on the left for the ovary, and a vas on the right for the testis. Shattock and Seligmann's bird had two vasa.

In comparing the structure of the reproductive organs, there again seems to be a preponderance of female over male. Four of Brandt's birds had ovaries in more or less embryonic or degenerate condition. The four mule pheasants of Smith and Thomas had ovaries composed of stroma and interstitial cells, with no oocytes. Nos. 1427, 1425 and 1428 of our birds belong to this type. One of Brandt's birds and 1429 of this paper had normal ovaries with oocytes. An ovotestis or mixed gland was present in two of Brandt's birds, in Shattock's Leghorn, in Bond's pheasant, and in Atwood's Hermaphrodite, 1349, and the Michigan bird of the present study. No. 1426 and Pearl and Curtis's bird No. 16 both had an ovary on the left and a testis on the right, with the difference that while in both, the ovaries were inactive, the testis in 1426 was active and that in 16 was not. One bird described by Brandt had two testes, one on each side. Hammond Smith is also quoted by Bond as describing three birds with some female secondary sex characters with normal testes present. In most of these birds, previously described, the gonad of whichever sex showed no signs of activity of the germ cells. The first of Brandt's birds is the one possible exception on the female side. Several of the birds in this study, however, show signs of past or present

activity of the ovary. No. 1429, Atwood's bird, and the Michigan bird have many oocytes of varying sizes on the periphery. No. 1349 and 1426 have some cystic oocytes, while there are corpora lutea representing discharged or atretic follicles on four birds, 1426, Atwood's bird, 1349, and the Michigan bird. No. 1429 has no discharged follicles, nor any very small oocytes. No signs of ovarian activity, either past or present could be discovered in the other three birds, 1428, 1427, 1425.

The only birds with active testis in which sperm were observed are Bond's pheasant, 1426, 1349, and 1616 described in this paper, possibly also the three birds of Hammond Smith, although we do not know how carefully the histology of these was studied. It should be noted that all eight birds described by the present authors may be interpreted as fundamentally females, some of them checked in the embryonic condition of the gonads, and some of them changing over to a male condition.

Next in the series of abnormal birds we have placed the guinea chicken hybrids. The anatomy of these is apparently that of perfectly normal males with two testes and two vasa deferentia. These were of normal size in one, and much enlarged in the other. That these testes, however, are not normal, is clearly shown in microscopic sections (fig. 31). There is no sign of tubules or any cells distinguishable as germ cells. The structure looks more like ovarian stroma than part of a testis, that is absolutely indifferent, whether ovarian or testicular in nature. In fact, it is probably neither, but simply an undifferentiated gonad, as in the early embryo. Neither are there any cells with the distinguishing marks of interstitial cells or luteal cells. This is, of course, an entirely different condition from that found in Guyer's guinea chicken hybrids, where there were many tubules and an abnormal synopsis either stopped the process of sperm formation or else resulted in abnormal spermatozoa. Poll has worked out the theory from his hybrid birds that the more closely related two crossed birds are, the more normal will be the spermatogenesis. This, however, does not explain how some male guinea chickens can have abnormal

sperm formed and others have no trace even of seminal tubules in their structure.

VI. DISCUSSION

The study of the anatomy and histology of this whole series of birds somewhat abnormal as to sex shows that they are all fundamentally female, except the guinea chickens. These are merely sterile males with consequent indifferent behavior. The hens with a tendency to tread other hens are normal active females. The eight other birds are fundamentally female, either undeveloped or degenerating. Every bird has in its embryonic development an undifferentiated sex stage as far as organs are concerned. It has the ducts for both sexes, and the gonad has the same early development regardless of whether it develops later into an ovary or testis. This undifferentiated gonad consists of a mass of sex cords growing out from the mesonephros covered over by a thickened germinal epithelium. If the bird becomes a female, the left Müllerian duct enlarges and the germinal epithelium proliferates and forms most of the reproductive organ. If the bird becomes a male, the Müllerian ducts degenerate, the Wolffian ducts become larger and coiled, and the sex cords become the main part of the reproductive organ. One of the Holland birds, 1429, is nearly a normal female. Three of the Holland birds, 1428, 1425, 1427, are evidently undeveloped females. They have infantile oviducts and embryonic ovaries. The other four birds are also fundamentally female, but show that the reproductive apparatus, has passed through a female stage and has become partly or largely male. In 1426, the ovary is partly embryonic, and partly degenerating, and a testis with active sperm has formed on the right side of the body. It has the oviduct of a laying hen. The other three birds have large gonads on the left side only and in all three cases the ovarian portion shows signs of degeneration and the testis portion signs of development. A transformation such as this can be easily understood from the embryonic development. The sex cords in the core of the gonad hypertrophy and form seminal tubules, while the oocytes and

follicles in the germinal epithelium are crowded to the edge of the organ and degenerate. The primary cause back of this shifting of development we make no attempt to explain at present.

As to the relation of the secondary sex characters to the primary sex organs, there is shown by this study only a very general correspondence such as in body shape and carriage. Spurs, comb and wattles vary regardless of primary sex organs. The general correspondence might be accounted for in accordance with the theory that the ovary forms an external secretion that inhibits maleness. In the cases where the ovary is embryonic it has not matured sufficiently to form such a substance. In the cases where it has degenerated its influence is past. That would account for the fact that birds with both embryonic and degenerating ovaries exhibit some male characters.

The interstitial cells are clearly shown to have nothing to do with any of the secondary sex characters. Their distribution is not correlated with any such characters. The luteal cells, however, are found to be in distinct and definite correlation with the degree of external somatic femaleness. Even in these abnormal ovaries, they seem to keep to their normal process of development, fill up the atretic and discharged follicles and finally form the characteristic yellow pigment. Just as Pearl and Surface showed to be the case in the cow, so here in fowls the degree to which an individual remains somatically female, is precisely reflected in the amount of luteal tissue in the ovary, and vice versa.

The behavior of these birds presents some distinct anomalies. It does not entirely correspond to the external characters nor to the stage of development of the gonads. The behavior of the birds with embryonic gonads is indifferent, although the birds show some adult external sex characters. No. 1426 has mature sperm, but entirely indifferent sex behavior. However, 1349 and 1616 show the same changing sex behavior as they do external characters and internal structure.

A fuller discussion of the facts of normal and abnormal sex structures and behavior, with their bearing on theories of sex and secondary sex characters will be presented in a later paper.

VII. SUMMARY

1. The eight hermaphrodite birds studied are females with embryonic or degenerating ovaries.
2. Three of these birds were changing from a female to a male condition in respect to internal structure (gonads), external characters and sex behavior.
3. There is no structural counterpart for the abnormal behavior of one hen treading another hen.
4. The two guinea chicken hybrids studied had testes composed of undifferentiated tissue. This is a different condition from that found by Guyer in a guinea chicken hybrid, and therefore calls in question Poll's theory that the stage of germ cell development in hybrids depends on the closeness of the relation of the individuals crossed.
5. Development of comb, spurs, and wattles does not stand in direct quantitative relation to the sex of the gonad.
6. Body shape and carriage, have a general relation to the sex of the gonad.
7. The interstitial cells clearly have no causal relation to the secondary sex characters in the abnormal birds here described.
8. Amount of luteal cells or pigment is in precise correlation with the degree of external somatic femaleness exhibited by the individual.

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PLATE 1

EXPLANATION OF FIGURES

- 1 Holland bird 1429.
- 2 Holland bird 1428.
- 3 Holland bird 1427.
- 4 Holland bird 1426.
- 5 Hermaphrodite 1349.
- 6 Michigan hermaphrodite 1616.



PLATE 2

EXPLANATION OF FIGURES

7 Dissection of urogenital system in a normal hen. *d*, oviduct; *o*, the ovary; *u*, a ureter; *v*, a vas deferens.

8 Section of ducts entering cloaca in a normal hen. *a*, is artery, *u*, the ureter, *v*, the vas deferens, or Wolffian duct.

9 Same as *v* in figure 8, showing columnar epithelium of the vas in a normal hen.

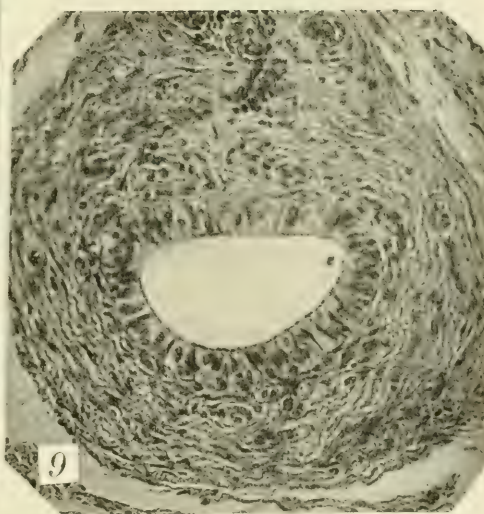
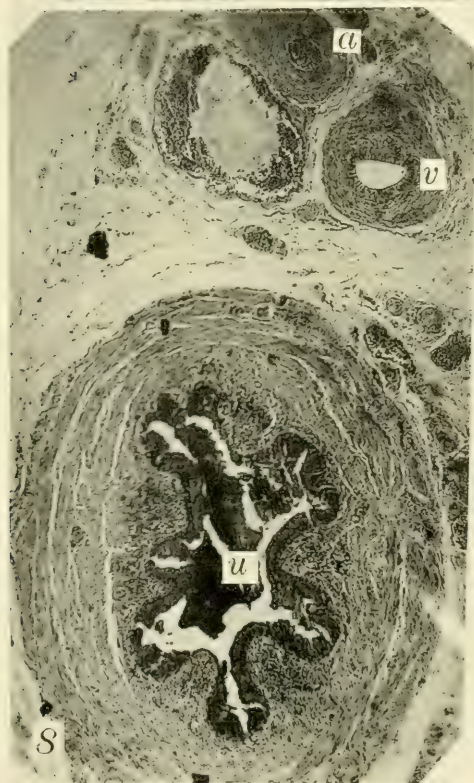


PLATE 3

EXPLANATION OF FIGURES

10 Dissection of urogenital system of bird No. 1429. *d*, the oviduct; *o*, the ovary; *r*, the rectum; *t*, the large tumor.

11 Dissection of urogenital system of bird No. 1428. *d*, oviduct; *g*, the gonad; *t*, the tumor.

12 Dissection of urogenital system of bird No. 1427. *d*, the oviduct, *g*, the gonad; *t*, the tumor.



PLATE 4

EXPLANATION OF FIGURES

13 Dissection of urogenital system of bird No. 1425. *d*, the oviduct; *g*, the gonad.

14 Dissection of urogenital system of bird No. 1426. *d*, the large coiled left oviduct; *o*, the ovary on the left; *rd*, the crumpled right oviduct; *ts*, the testis lying on the right.

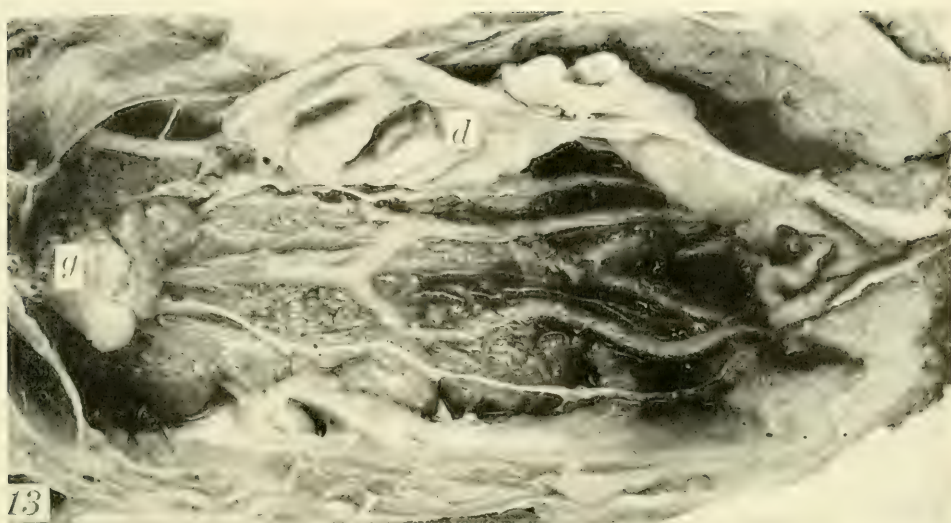


PLATE 5

EXPLANATION OF FIGURES

15 Dissection of the urogenital system of Atwood's hermaphrodite bird. *d*, the small oviduct; *o*, the ovarian part of the left gonad; *ts*, the testicular part of the gonad.

16 Dissection of reproductive system of bird No. 1349. *d*, the coiled oviduct; *g*, the reproductive organ.

17 Dissection of urogenital system of the Michigan hermaphrodite, No. 1616. *d*, the large oviduct; *g*, the large reproductive organ with a few oocytes showing on the surface.

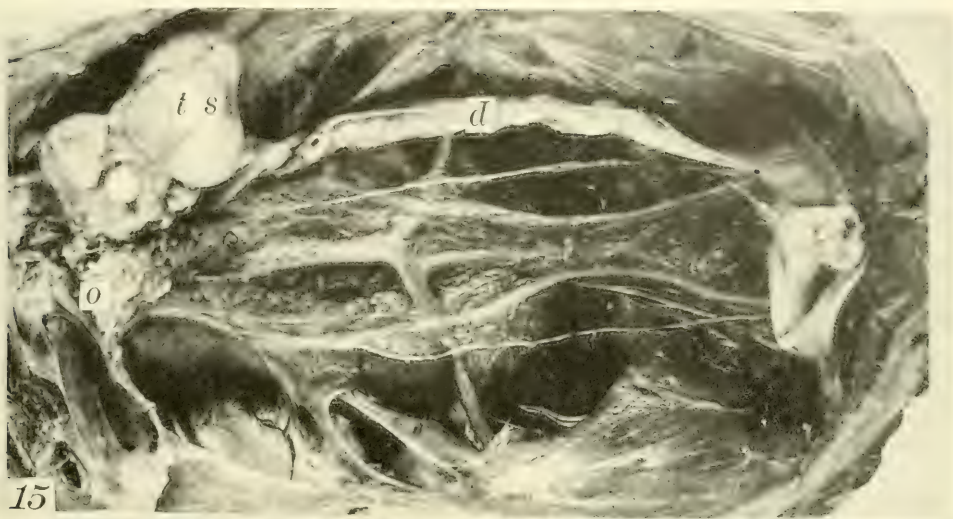


PLATE 6

EXPLANATION OF FIGURES

- 18 Section of ovary of 1429 showing oocytes ($\times 40$).
- 19 Section of ovary of 1428, showing stroma and mesonephric tubules ($\times 80$). *t*, group of luteal cells.
- 20 Section of another part of gonad of 1428, showing mesonephric tubules and groups of luteal cells (*t*). $\times 176$.
- 21 Section of ovary of 1427, composed of nothing but stroma. ($\times 80$).

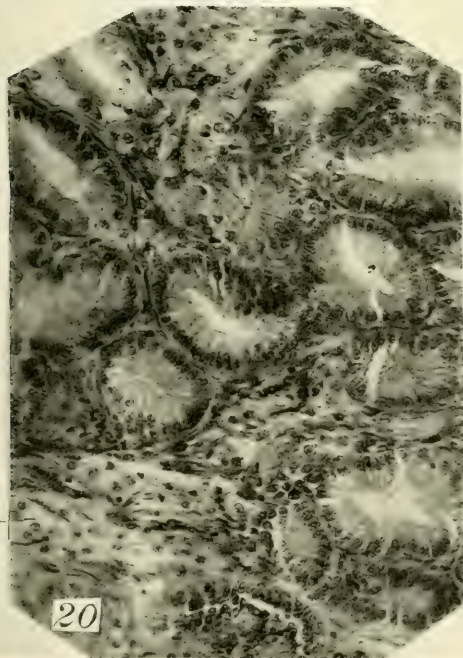
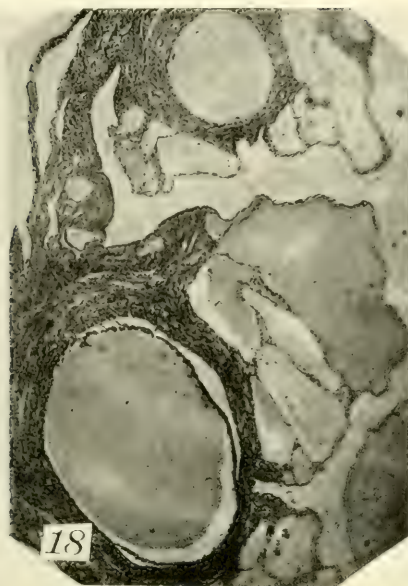


PLATE 7

EXPLANATION OF FIGURES

- 22 Section of ovary of 1425, showing tubules and germinal epithelium ($\times 80$).
- 23 Section of testis on right side of 1426, showing seminal tubules ($\times 40$).
- 24 Part of figure 23, showing two seminal tubules with sperm in center ($\times 176$).
- 25 Section of ovary on left side of 1426, showing stroma ($\times 80$).

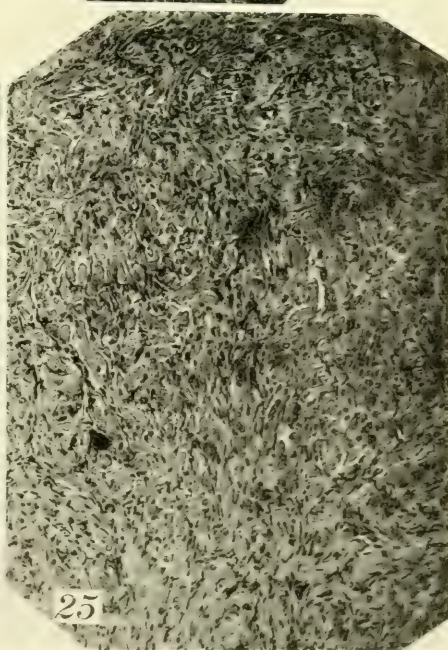
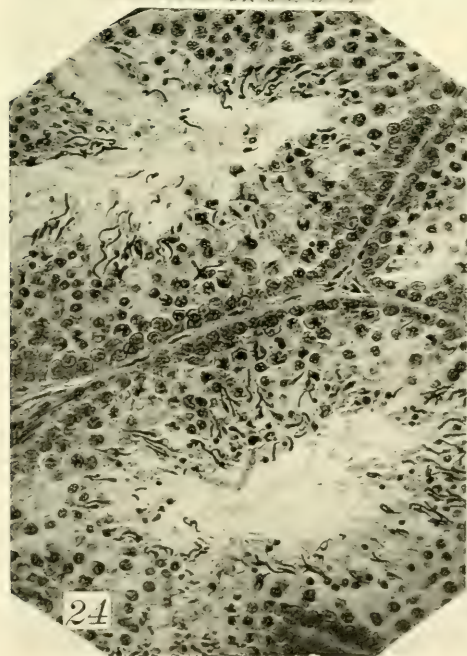
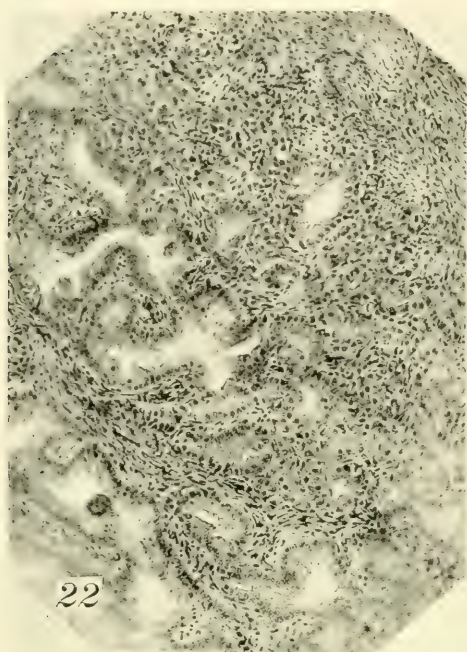


PLATE 8

EXPLANATION OF FIGURES

- 26 Section of ovarian part of gonad on left side of Atwood's hermaphrodite ($\times 40$). *ts*, the testis tubules in the center of the organ.
- 27 Section of very small testis on right side of Atwood's hermaphrodite ($\times 80$).
- 28 Section of testis part of gonad of 1349 ($\times 40$).
- 29 Part of figure 28, showing seminal tubules with spermatids in center ($\times 176$).

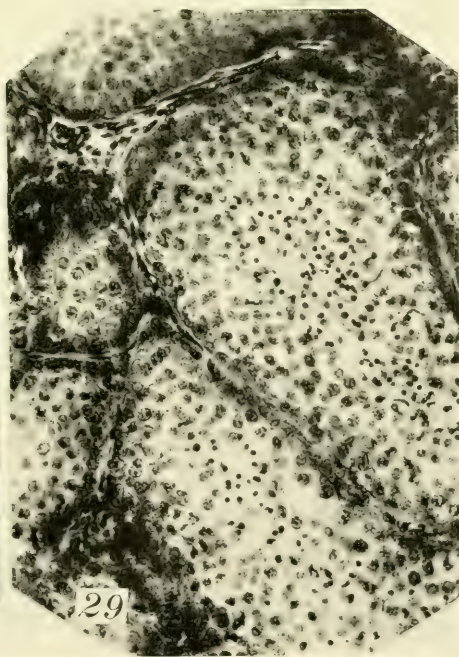
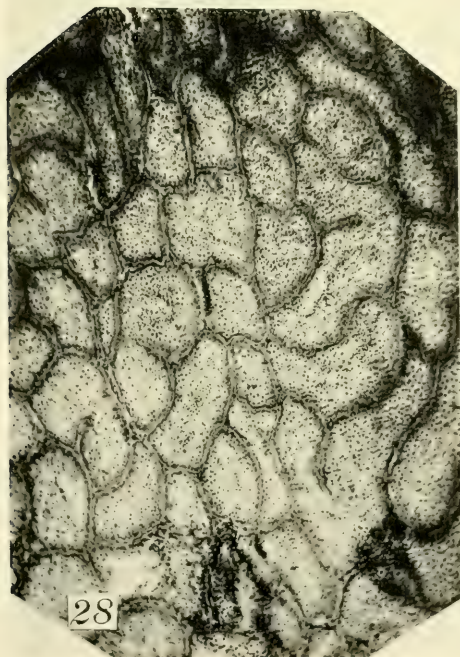
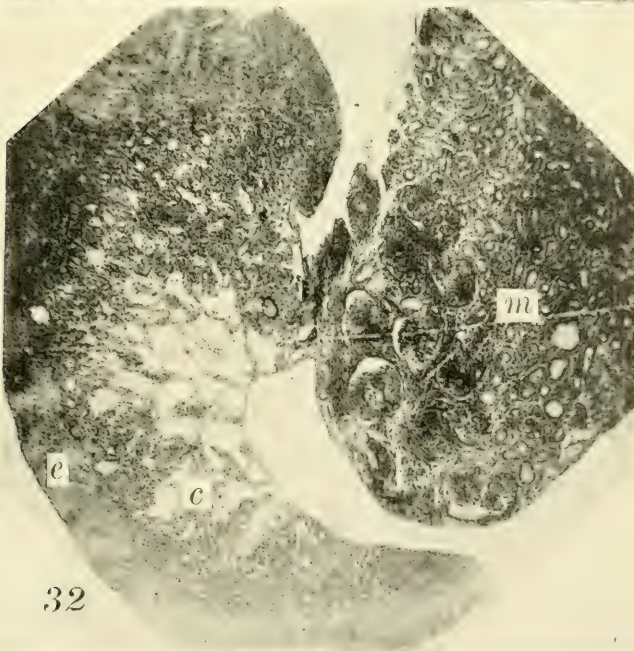
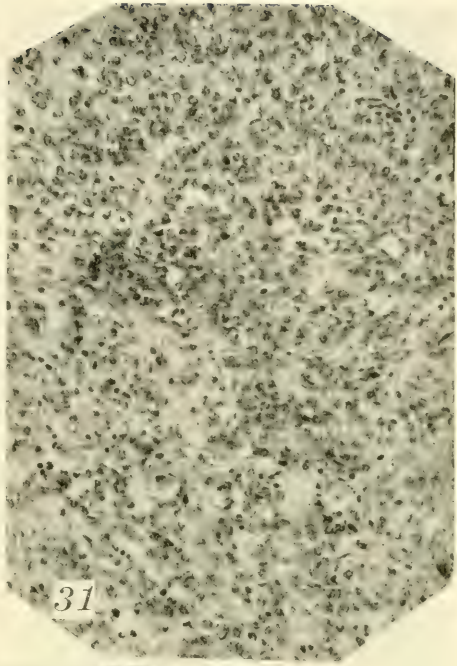
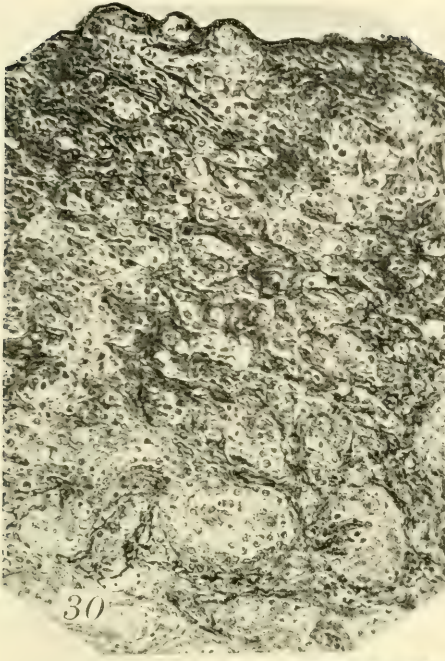


PLATE 9

EXPLANATION OF FIGURES

- 30 Section of an ovarian part of gonad of 1349, showing stroma ($\times 80$).
- 31 Section of gonad of a guinea chicken hybrid, showing that organ is composed entirely of stroma ($\times 176$).
- 32 Section of ovary of a just-hatched chick ($\times 40$). *c*, part composed of sex cords; *e*, germinal epithelium. *m* is mesonephros.



THE REACTIONS TO LIGHT AND TO GRAVITY IN DROSOPHILA AND ITS MUTANTS

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THREE FIGURES

INTRODUCTION

The presence in the Columbia laboratory of a great variety of mutant stocks of *Drosophila ampelophila* involving differences in the size of wings and color of eyes has offered an exceptional opportunity to investigate the relation of these variations to the responses of the fly to light and to gravity. The wing mutations, moreover, have been useful in checking up results obtained by removal of part of the normal wings, while the differences in eye color have made possible a novel series of experiments on the effects of lights of varying wave lengths.

Finally, in one case it has been possible to study the direct hereditary modification of a reaction system apart from any significant morphological variation.

Although a considerable amount of work on the behavior of *Drosophila* toward light and gravity has already been done, notably by F. W. Carpenter, W. H. Cole, Fernandus Payne, A. O. Gross and F. E. Lutz, it was nevertheless found necessary to try out a number of preliminary experiments in order to determine the source of certain discrepancies in the results of these investigators. This was particularly true in regard to the effect of the factors of age and sex on normal flies.

I wish at this point to express my sincere thanks to Dr. T. H. Morgan for his many helpful suggestions in the course of my experiments. I also wish to express my indebtedness to Dr. Francis H. Herriek for allowing me the use of the laboratory at Western Reserve University during the summers of 1915 and

1916. Finally I am under great obligation to Dr. W. L. Severinghaus and Mr. E. C. Unnewehr, who, under the direction of Dr. Trowbridge determined for me the wave lengths and energy transmitted by my colored filters. This work was done in the Ernest Kempton Adams Precision Laboratory of Columbia University.

METHOD AND APPARATUS

A variety of methods were employed in the course of the work, and it therefore seems unprofitable at this point to do more than give a general description of the apparatus and the chief systems used in making the tests.

A glass tube 26 mm. in internal diameter and 91.4 cm. long contained the flies. This tube was graduated in inches because at the beginning this seemed to be about the smallest unit in which it was possible to observe rapidly moving flies with any degree of accuracy. One end of the tube was closed by a piece of isinglass, while the other was stopped with a cork covered with dead-black paper. The flies were introduced into the tube by means of a cardboard funnel from small vials in which they were kept. The tube was then placed with the open end toward a window in a room which was otherwise darkened. Since it was early discovered in accordance with the results of Carpenter (*American Naturalist*, '05) that the insects were much more phototropic when mechanically stimulated, the tube was held in the hands during the earlier trials and gently agitated.

After a short series of experiments under these conditions it became evident that a more refined system must be used in order to get results in any way comparable or consistent. The two chief factors which demanded standardization were the means of mechanical agitation and the source of light. The first problem was solved as follows. The tube was fastened in a horizontal position on a board by means of rubber bands and nails padded by thick felt. The four nails were placed in pairs, each pair about 22 cm. from the end of the tube, in such a way that the latter could vibrate between the nails in a direction at right angles to its length and through a distance of about 5 mm. A pendulum was now constructed from a piece of wood a meter in

length, to the end of which was fastened an oblong piece of lead weighing 410 grams and heavily padded with felt. This pendulum was then suspended in such a manner that at the end of each complete vibration the padded end would strike the tube at its middle point. The length of the swing was limited by the tube, upon the one hand, and a shelf against which the pendulum hit on its backward stroke. This shelf was back of the tube a horizontal distance of 50 cm. and 22 cm. above it. When the tube was placed in position for operation, it was held in place by the rubber bands mentioned above. These bands were fastened to a couple of tacks on the side of the tube toward the pendulum and at equal distances from its center. These were then passed under the tube toward the operator, and then over it to the edge of the board toward the pendulum, where they were again fastened. Thus when the pendulum was set in motion it would strike the tube and push it a slight distance away from the nails against which the bands tightly held it. These would immediately snap it back into position ready to be again displaced by the next swing of the pendulum. Thus the tube underwent a constant jarring of a fixed degree of violence and at regular intervals. The pendulum was kept in motion by a slight pressure of the hand on its upper end, delivered just at the beginning of each backward swing. While it might be objected that this pressure would vary, thus varying the length of the stroke and the force of the blow, this variation in practice was found to be very slight indeed. This was made possible by the fact that great care was used to give exactly the amount of pressure necessary to make the pendulum just reach the shelf on its backward swing. With a little practice this action became mechanical and exceedingly constant. The degree of accuracy was in fact determined thus. An assistant counted the number of strokes per minute for five separate trials with an interval of a minute and a half between each trial. The count each time was 40 strokes. The strokes were then counted for three consecutive minutes, and the count was 118. According to the other test it should, of course, have been 120, thus showing the very small error of 0.6 of a stroke per minute. No greater care was exercised during these tests than was normally the case in the experi-

ments, and while it is possible that the desire for a certain outcome might cause an unconscious variation at times, I think it very unlikely that this was of sufficient magnitude to seriously alter the results.

For the purpose of providing a constant standard source of light a 200 watt nitrogen filled Mazda lamp was suspended within a tin box 24 cm. high by 14 cm. wide and 14 deep. Small holes were made in the bottom and the top of this box to allow for ventilation, while in its front, just opposite to the concentrated filament of the lamp, was cut an aperture 3.5 cm. in diameter. In front of this opening was fastened a flat flask containing water. A second box of the same dimensions was now fitted to the front of the first and in its front a hole was cut equal in size to the hole in box number one and exactly on a line with it. This aperture was fitted with clamps for holding the flasks which were to contain colored liquids when such were desired. If only white light was wanted the outer as well as the inner flask contained clear water. This arrangement of two flasks not only shielded the insects from heat rays, but also prevented the colored liquids from becoming hot. The flasks used were about 1.5 cm. thick, and identical flasks were employed in the same positions throughout the experiments.

This apparatus was set up at the end of the table on which the testing tube was fastened, in such a manner that the center of the lamp filament was exactly opposite to the end of the tube and about 24 cm. away from it. This made the end of the tube about 3 cm. from the outer flask. Finally a blackened screen was set up between the end of the tube and the light boxes, with an aperture in it opposite to the end of the tube and about the size of the flasks. The purpose of the screen was to shade the tube from the slightly diffused light which escaped from cracks in the boxes and through the holes above the lamp. The room where most of the experiments were performed had its walls painted a dead black, and the windows were provided with black opaque shades. All timing was done with a good watch placed in such a position that the face was illuminated by light issuing from the aperture. A diagram of this apparatus is shown in figure 1.

METHODS USED FOR MEASURING REACTIONS

The following were the chief systems devised for measuring the phototropism of flies with the above apparatus. The insects to be tested were taken from the breeding bottle and etherized. Those to be tested were then isolated and placed in small vials with or without food, according to the detailed conditions of the particular experiment. When about to be tested, unless otherwise indicated, the flies were always light adapted, after which they were introduced into the large testing tube. At first this was done by means of a funnel, but later on the vials were

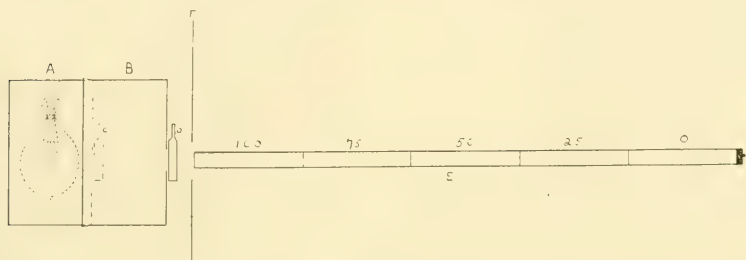


Fig. 1. *A*, First box containing 200 watt lamp. *B*, Second box to the front of which is fastened the outer flask *D*, containing either clear water or colored liquids. *C*, Inner flask fastened to front of first box, and always containing clear water. *E*, Tube divided into fifths valued as indicated for the purpose of calculating the tropic indices. *F*, Black screen to shield tube from stray light rays.

padded so that the mouths would just fit into that of the tube, into which the insects were allowed to crawl with as little shaking as possible. When the flies had been attracted into the proper end the tube was slipped into position and the test begun. If agitation was desired, a lever which released the pendulum was struck the instant that the tube was in place. When the test in question was completed the flies were replaced in their original position as follows. The tube was taken before a window or bright lamp and gently tapped with the fingers or twirled between them. If this method failed, as in the case of non-phototropic flies, shaking was resorted to. Variations of this sort will be mentioned in connection with the experiment under discussion.

There were two chief methods used for measuring the response. In the first of these, one fly was tested at a time. It was placed in the end of a tube away from the light, and allowed to remain for one minute. During that minute a record was taken of the furthest distance which it crawled toward the light, expressed in inches. If it had not reached the light end, it was now put there by the method described above, and the test repeated. This time, however, the furthest inch which it crawled away from the light was recorded. Each of these tests was repeated three times, the ends of the tube in which the insects started being alternated. An average for the inches crawled toward the light in each trial was then taken and an average of the inches crawled away from it.¹ For convenience, the former will be referred to as the t. l. (toward light) average and the latter as the f. l. (from light) average. The algebraic sum of these two averages was taken as the t. l. or f. l. index of the fly in question. In any given experiment as many flies as possible were thus tested and the sums of the t. l. indices algebraically added to the sums of the f. l. indices. Thus, let us suppose that there were four flies in a group. The index of fly 1 was t. l. 20, that of fly 2, t. l. 30, that of fly 3, f. l. 10, and that of fly 4, f. l. 5. The index for the group would be expressed as t. l. 35. This method will be designated as method I.

After using this method for some time it became evident that a plan must be devised for testing more flies at a time. This seemed desirable because of the extreme variability of individuals, despite all efforts to keep environmental factors constant. The following plan was worked out for obtaining the phototropic index of insects by groups. This system proved far more satisfactory than the one described above and has been used in all the later and more critical work. It will be designated as method II.

The tube in this case was divided into fifths, and to each fifth was assigned an arbitrary value as follows. The division fur-

¹ This means of expression is necessary in order to avoid the use of the term negative. The fact that the insects crawl away from the light end does not prove them negative, since when placed in that end they have no other choice. Furthermore, other results make it unlikely that the flies ever give a truly negative reaction.

thest from the light counted zero, the second 25, the third 50, the fourth 75 and the last which came nearest the light, 100. A given number of flies, usually ten, was introduced into the zero end and the test lasting one minute was begun. During the minute a record was taken of the furthest sections reached by any of the flies. The number of flies in each section was then multiplied by the value assigned to the section in question. The total number of values thus obtained were then added together and divided by the number of flies used. Thus, let us suppose that ten flies were undergoing a test. Eight flies reached the section nearest the light valued at 100. Two flies only reached the second section valued at 25. The process for obtaining the phototropic index was as follows. $100 \times 8 = 800$, $25 \times 2 = 50$, $800 + 50 = 850$. Divide by 10 the number of flies and we have 85. This, then, will be the phototropic index for these flies in this test. It will be noted that there is not in this method any means of detecting actually negative flies. When this system was started, however, enough work had already been done to show that none of the insects worked with were actually negative under any circumstances. The problem had therefore resolved itself into one of determining the relative positivity of the various types experimented with, and for that purpose the system just described was superior to the first.

Finally, it is evident that virtually the same general method could be used in studying the relative reactions of different groups of flies to gravity. The detailed changes introduced in connection with the gravity experiments as well as those employed in some of the special light tests will be dealt with in the description of the particular experiment in question.

EFFECTS OF AGE AND SEX

In a paper entitled "Biological Notes Concerning *Drosophila ampelophila*," published in the Journal of the New York Entomological Society for June, 1914, vol. 22, F. E. Lutz of the American Museum of Natural History, states that *Drosophila* shows the maximum phototropic response at the age of 18 hours, after which the reaction gradually diminishes in intensity. He also

states that the females are nearly twice as responsive as the males. Concerning the latter point Moenkhaus has reached exactly the opposite conclusion, while the writer's early work failed to confirm the former. It therefore became necessary to settle these questions before reliable results could be obtained in which other factors were involved.

After a number of preliminary trials the following experiment was devised to test thoroughly the effect of age and sex upon the insect's light reaction. Incidentally the influence of these factors upon fatigue is also brought out. Method II was used for measuring the response of the flies by groups, while mechanical agitation by means of the pendulum was employed throughout the experiment. The animals were introduced into the tube from the padded vials as described above, with as little disturbance as possible. They were returned to the starting point between each test by means of light, with only such slight shaking as was absolutely necessary to get them in place inside of a minute and a half after the preceding test. Usually very little was required. The groups of flies to be tested were removed from the culture bottles late in the afternoon, and placed in vials with a small quantity of food. The females were a trifle older than the males in most cases and have been designated as 17 to 19 hours, while the males were recorded as 16 to 18. The test was always made the following morning, beginning at nine o'clock. The males were given 32 successive tests while the females received 50. As indicated above, there was an interval of a minute and a half between each test during which the flies were transferred to the starting point by taking the tubes to a window and allowing them to crawl toward the light. After this series of experiments was completed, a second series was run with flies from 4 to 6 days old. In the case of groups A, B, C, D, and E of this series, the insects had not been tested before, but groups F, and G were flies which had been tested in the first series under groups D and E respectively. Thus in these two instances it is possible by reference to the tables to get a direct comparison between the reactions of the same flies at different ages. Suffice it to say that the results are in entire accord with those obtained by averag-

TABLE 1

Average indices of male flies 4 to 6 days old

| | | | | | | | | | | |
|------------|------|------|------|------|------|------|------|------|------|------|
| Tests..... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Indices... | 78.9 | 87.3 | 93.2 | 91.9 | 96.8 | 93.7 | 94.9 | 91.5 | 92.2 | 94.0 |
| Tests..... | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Indices... | 95.9 | 95.5 | 92.3 | 89.7 | 91.4 | 93.2 | 90.6 | 92.3 | 87.0 | 88.8 |
| Tests..... | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Indices... | 90.1 | 88.3 | 88.8 | 82.4 | 84.8 | 85.5 | 84.7 | 85.6 | 85.5 | 84.3 |
| Tests..... | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| Indices... | 87.0 | 83.1 | | | | | | | | |

Average indices of female flies 4 to 6 days old

| | | | | | | | | | | |
|------------|------|------|------|------|------|------|------|------|------|------|
| Tests..... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Indices... | 91.5 | 97.7 | 95.8 | 94.9 | 95.4 | 94.1 | 96.2 | 94.9 | 90.6 | 94.3 |
| Tests..... | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Indices... | 88.2 | 92.2 | 91.0 | 90.0 | 92.3 | 90.5 | 84.3 | 88.8 | 89.7 | 86.1 |
| Tests..... | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Indices... | 83.3 | 84.7 | 89.2 | 86.5 | 91.0 | 87.0 | 85.2 | 83.9 | 86.4 | 75.8 |
| Tests..... | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| Indices... | 78.9 | 74.5 | 84.3 | 79.8 | 74.5 | 75.8 | 84.1 | 72.2 | 79.4 | 77.6 |
| Tests..... | 41 | 42 | 42 | 44 | 45 | 46 | 47 | 48 | 49 | 50 |
| Indices... | 78.9 | 77.6 | 75.8 | 70.9 | 76.7 | 80.3 | 79.7 | 72.7 | 76.7 | 62.4 |

Average of male flies 16 to 18 hours old

| | | | | | | | | | | |
|------------|------|------|------|------|------|------|------|------|------|------|
| Tests..... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Indices... | 72.4 | 78.5 | 83.1 | 80.5 | 80.5 | 76.8 | 76.2 | 72.4 | 72.4 | 68.7 |
| Tests..... | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Indices... | 67.4 | 77.4 | 71.6 | 71.2 | 67.4 | 71.2 | 66.2 | 68.7 | 65.6 | 61.2 |
| Tests..... | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Indices... | 64.9 | 61.8 | 58.7 | 57.4 | 56.2 | 52.4 | 56.8 | 49.9 | 54.3 | 46.8 |
| Tests..... | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| Indices... | 44.9 | 46.8 | | | | | | | | |

Average of female flies 17 to 19 hours old

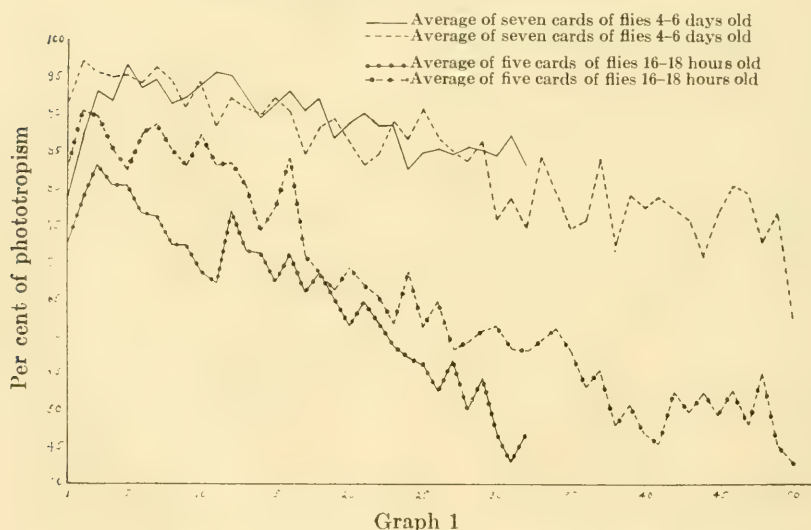
| | | | | | | | | | | |
|------------|------|------|------|------|------|------|------|------|------|------|
| Tests..... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Indices... | 83.0 | 90.6 | 89.9 | 85.5 | 82.4 | 87.4 | 88.7 | 85.6 | 83.1 | 87.4 |

TABLE 1—Continued

| | | | | | | | | | | |
|------------|------|------|------|------|------|------|------|------|------|------|
| Tests..... | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Indices... | 83.1 | 83.7 | 80.5 | 74.1 | 77.4 | 84.3 | 71.2 | 68.7 | 66.2 | 69.3 |
| Tests..... | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Indices... | 66.8 | 65.6 | 81.8 | 68.7 | 61.2 | 64.9 | 58.7 | 59.3 | 60.6 | 61.6 |
| Tests..... | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| Indices... | 58.1 | 58.0 | 59.3 | 61.2 | 58.0 | 53.1 | 55.5 | 48.0 | 50.6 | 46.8 |
| Tests..... | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 |
| Indices... | 45.6 | 52.4 | 49.9 | 52.4 | 49.3 | 52.9 | 48.1 | 55.3 | 45.6 | 43.0 |

ing all the groups in each series. This was done by averaging the corresponding tests in each group of the same age and sex (table 1, graph 1).

Fatigue curves for flies of different ages



It appears that the younger flies are rather less phototropic, or at least less active, than those 4 or 5 days old. Also they tire more easily. Furthermore, although in the younger groups the females are consistently above the males, in the older groups the males, after the first few tests are quite the equals of the females. This result is substantially in accord with that obtained in my preliminary tests.

As has been noted, the older females gain rather less relatively in speed than the older males. This, it appears, might very likely be due to the fact that the female is gradually weighed down by the growth of her eggs. Indeed, it is known that these do not reach their full size for 4 or 5 days, the time varying somewhat with the abundance of food. This suggested the experiment of running some insects of both sexes through a series of tests to be made daily for a period of two weeks, during which time they would be given fresh food each day. Three groups of flies with ten males and ten females in each group were selected for this purpose. Five trials were given to every set of ten flies at each of the testing periods, and the results of these five trials averaged for the period in question. This average has been taken as the index for the set of ten flies for this test. It is from an average of these averages that table 2 is made up. The tests for the first day were given at the ages of 4, 12, 15, 18, 21, and 24 hours. Afterward there was one test a day consisting of the usual five trials. The results indicated in table 2 and the corresponding graph show that the females, though starting ahead of the males, fell away much more rapidly than usual, thus tending to confirm the conclusion that a good share of their falling off with age is due to the increased weight of their ovaries. Incidentally, it is again evident that the strongest reaction does not come at 18 hours.

The next step was to run a similar series of daily tests for flies whose food was not changed daily. In this case a small amount of banana food was put in the vial in which a group of flies was kept and allowed to remain there. It gradually dried up so that the flies could derive less nourishment from it each day. That they must have derived some is certain, for *Drosophila* can not live 24 hours without food. Besides the drying, however, there is also a chemical change in food in which larvae are not working. This, as well as the drying, tends after a few days to make the food unfit for the insects. This fact accounts for the death of those flies to which a little fresh food was not given on the sixth day. From this, as well as other experiments, it appears that 6 days is about the average time that flies will live under

such conditions, though I have not infrequently had them live longer. For some reason not recorded no B and C groups of males were started. Hence this experiment is not particularly satisfactory. However, so far as it goes it tends to uphold the views already stated. The males gain on the females as age increases, and both sexes show a general increase up to the time when feeding was necessary. The results are summarized in table 3 and the accompanying graph.

There remain to be described a couple of experiments which throw more light on the fatiguing effect of frequent tests given

TABLE 2

Males

| Temperature..... | 22° | 20° | 21.5° | 23.2° | 24° | 24.1° | 23° | 24° | 22.5° |
|--------------------------|-------|------|-------|-------|------|-------|------|------|-------|
| | Hours | | | | | | Days | | |
| Age in hours or days.... | 4 | 12 | 15 | 18 | 21 | 24 | 2 | 3 | 4 |
| Group: | | | | | | | | | |
| A..... | 82.5 | 60.5 | 57.5 | 62.0 | 69.5 | 72.5 | 71.0 | 84.0 | 83.0 |
| B..... | 73.5 | 53.5 | 57.5 | 54.5 | 75.5 | 68.0 | 72.0 | 81.5 | 77.5 |
| C..... | 83.5 | 51.5 | 62.5 | 63.0 | 87.5 | 89.5 | 95.0 | 96.0 | 92.0 |
| Average..... | 79.8 | 55.1 | 59.1 | 59.8 | 77.5 | 76.6 | 79.3 | 87.1 | 84.1 |

| Temperature..... | 21.0° | 21.6° | 23.5° | 24.6° | 22.8° | 21.5° | 22.5° | 20.0° | 23.0° | 24.5° |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|-----------------|-------|
| | Days | | | | | | | | | |
| Age in days..... | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 ¹ | 14 |
| Group: | | | | | | | | | | |
| A..... | 69.5 | 79.3 | 66.6 | 87.7 | 73.2 | 62.1 | 53.8 | 47.1 | 81.2 | 83.0 |
| B..... | 81.5 | 75.0 | 71.0 | 68.5 | 65.5 | 69.0 | 56.0 | 44.0 | 58.5 | 53.0 |
| C..... | 88.0 | 91.5 | 91.0 | 89.5 | 82.0 | 85.0 | 83.5 | 79.5 | 81.0 | 82.0 |
| Average..... | 79.6 | 81.9 | 76.2 | 81.9 | 73.5 | 72.0 | 64.4 | 56.8 | 73.5 | 72.6 |

¹ On this day one fly from the A group of males was lost. As the records indicate that very probably the fly lost was a slow one, a new calculation was made on the assumption that had this fly been present it would always have remained in the zero section. The figures resulting from this calculation are 72.1 for the thirteenth day and 73.8 for the fourteenth. This changes the averages for these days to 70.5 and 69.6 respectively.

TABLE 2—Continued.
Females

| Temperature.... | 22° | 20° | 21.5° | 23.2° | 24.0° | 24.1° | 23.0° | 24.0° | 22.5° | 21.0° |
|---------------------------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|
| Age in hours or days..... | Hours | | | | | | Days | | | |
| | 4 | 12 | 15 | 18 | 21 | 24 | 2 | 3 | 4 | 5 |
| Groups: | | | | | | | | | | |
| A..... | 92.5 | 69.0 | 90.0 | 76.5 | 84.0 | 84.0 | 75.5 | 76.5 | 70.5 | 72.0 |
| B..... | 95.0 | 79.5 | 77.0 | 77.5 | 90.0 | 87.0 | 83.0 | 83.0 | 76.5 | 65.5 |
| C..... | 94.5 | 73.0 | 70.5 | 78.5 | 97.5 | 92.0 | 85.5 | 81.5 | 75.5 | 61.5 |
| Average..... | 94.0 | 73.8 | 79.1 | 76.8 | 90.5 | 87.6 | 81.6 | 80.3 | 74.1 | 69.6 |

| Temperature..... | 21.6° | 23.5° | 24.6° | 22.8° | 21.5° | 22.5° | 20.0° | 23.0° | 24.0° |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Age in days..... | Days | | | | | | | | |
| | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Groups: | | | | | | | | | |
| A..... | 65.0 | 58.5 | 56.5 | 51.0 | 38.0 | 33.5 | 33.5 | 44.0 | 43.5 |
| B..... | 65.5 | 54.0 | 41.0 | 38.5 | 35.0 | 36.5 | 27.5 | 38.5 | 37.0 |
| C..... | 63.5 | 43.5 | 43.5 | 32.5 | 26.5 | 37.0 | 18.5 | 29.0 | 29.5 |
| Average..... | 64.6 | 52.0 | 47.0 | 40.6 | 33.1 | 35.6 | 26.5 | 37.1 | 36.0 |

Showing change in phototropism of wild flies when fed daily with fresh food

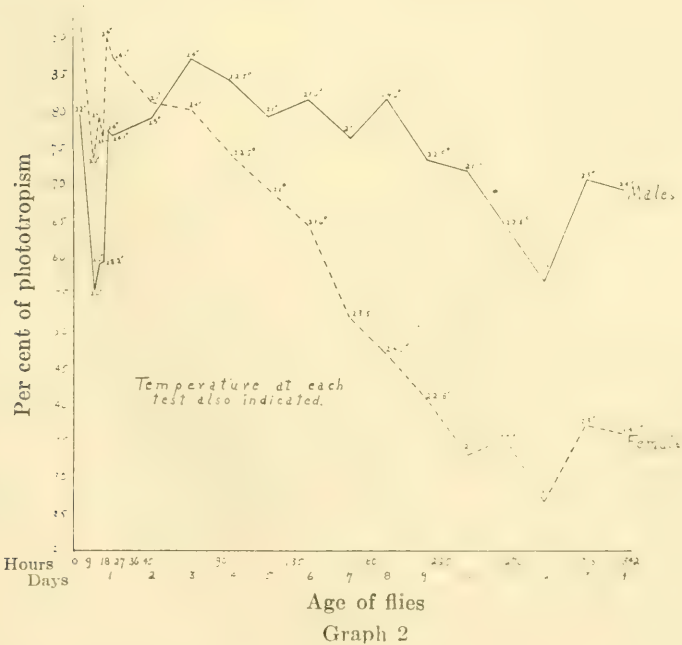


TABLE 3

Males

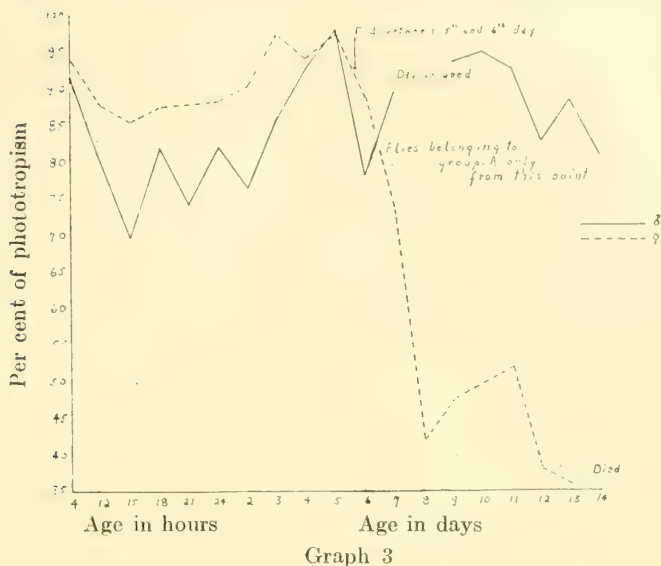
| Temperature..... | 22.7° | 21.5° | 21.2° | 21.5° | 22.2° | 24.5° | 23.5° | 21.6° | 21.1° | 22.1° |
|---------------------------|-------|-------|-------|----------------|-------|-------|-------|-------|-------|----------------|
| Age in hours or days..... | Hours | | | | | | Days | | | |
| | 4 | 12 | 15 | 18 | 21 | 24 | 2 | 3 | 4 | 5 ¹ |
| Group A: 7 flies..... | 91.4 | 80.6 | 69.9 | 81.3 | 74.2 | 82.1 | 76.3 | 85.8 | 92.8 | 97.8 |
| Temperature..... | 23.0° | 22.6° | 21.8° | 23.0° | 23.0° | 22.6° | 21.7° | 21.2° | 24.7° | |
| Age in days..... | Days | | | | | | | | | |
| | 6 | 7 | 8 | 9 ² | 10 | 11 | 12 | 13 | 14 | |
| Group A: 7 flies..... | 77.7 | 89.1 | | 93.3 | 94.9 | 92.4 | 82.4 | 88.3 | 80.1 | |

Females

| Temperature..... | 23.5° | 22.4° | 22.1° | 22.7° | 22.0° | 23.0° | 23.0° | 23.1° | 23.0° | 21.6° |
|---------------------------|-----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|----------------|
| Age in hours or days..... | Hours | | | | | | Days | | | |
| | 4 | 12 | 15 | 18 | 21 | 24 | 2 | 3 | 4 | 5 ¹ |
| 6 flies A..... | 80.8 | 70.8 | 76.6 | 72.4 | 64.9 | 68.3 | 85.8 | 96.6 | 93.3 | 97.4 |
| 9 flies B..... | 99.4 | 96.6 | 85.5 | 92.1 | 77.4 | 93.8 | 97.7 | 98.8 | 94.9 | 97.7 |
| 10 flies C..... | 100.0 | 96.5 | 95.5 | 98.0 | 95.3 | 98.0 | 87.5 | 96.0 | 94.0 | died |
| Average..... | 93.4 | 87.9 | 85.8 | 87.5 | 79.2 | 88.3 | 90.3 | 97.1 | 94.0 | 97.5 |
| Temperature..... | 21.2° (for B and C females only). | | | | | | | | | |
| Age in days..... | Days | | | | | | | | | |
| | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |
| 6 flies A..... | 81.6 | 73.3 | 41.6 | 47.4 | 49.1 | 51.6 | 37.4 | 35.8 | died | |
| 9 flies B..... | 96.0 | died | | | | | | | | |
| Average..... | 88.8 | | | | | | | | | |

¹ Indicates that food was changed at this point to prevent flies from dying. The drop on the sixth day is probably accounted for by this fact.

² Indicates only six flies in this group from the ninth day on.



Graph 3

to flies of different ages, as well as on the problem of time of maximum activity.

The first of these experiments was designed to show the effect of testing flies aged 18 to 24 hours three times with an interval of 2 hours between the tests. Seven groups of insects were thus tested, each group save three containing sixteen flies, eight male and eight female. Groups A and B only contained six flies of each sex, and Group E only five. The usual three trials constituted a test for each group, the sexes as always being tested separately. The average of the three trials is given as the index for the test in question.² The following summary of the results was obtained by averaging the indices of the seven groups (table 4).

Along with this series of tests there was run a parallel series similar in every respect except that the insects were 9 days old instead of 18 hours. Only six groups were used in this case, but there were eight flies in every group. A separate single

² It is to be noted that in this experiment as well as in the one on fatigue, the testing tube was only divided into four sections instead of five. The sections were then valued as follows: 100, 75, 25 and 0. With this variation calculations were made as described under method II.

TABLE 4

| | MALE | FEMALE | TEMPERATURE |
|--|------|--------|-------------|
| First test..... | 68.9 | 87.7 | 24.3° |
| Second test..... | 59.9 | 85.3 | 24.4° |
| Third test..... | 55.5 | 83.5 | 24.5° |
| Average..... | 61.4 | 85.5 | |
| Difference between males and females..... 24.1 | | | |
| Total number of males used..... | | | 47 |
| Total number of females used..... | | | 47 |
| Total..... | | | 94 |

group was put through the series at 6 days, and the results were practically similar to those obtained from the 9 day flies. For the sake of uniformity, however, they are not included in the following summary (table 5).

TABLE 5

| | MALE | FEMALE | TEMPERATURE |
|---|------|--------|-------------|
| First test..... | 71.6 | 78.3 | 25.1° |
| Second test..... | 74.8 | 83.9 | 25.4° |
| Third test..... | 76.8 | 83.3 | 25.5° |
| Average..... | 74.4 | 81.8 | |
| Difference between males and females..... 7.4 | | | |
| Total number of males used..... | | | 48 |
| Total number of females used..... | | | 48 |
| Total..... | | | 96 |

A glance at tables 4 and 5 is sufficient to indicate the general results. It again appears that the younger flies, both male and female, are fatigued by successive tests, whereas the older insects actually improve. Also it is clear that although the females are more active than the males in both cases, they are relatively less so in the older groups. Not only this, but the older females are absolutely slightly less active than are the females of the younger group. In this particular instance, therefore, 18 hours is actually the maximum age of activity. It may be added, however, that were the results from the 6 day flies

TABLE 6

| | MALE | FEMALE |
|------------------|------|--------|
| First test..... | 71.8 | 97.8 |
| Second test..... | 90.0 | 100.0 |
| Third test..... | 93.1 | 97.8 |

included in this average this statement would no longer hold. The indices for these insects were as follows (table 6): The average male index is 84.9 and the female, 98.5, with a difference of 13.6. Thus while the males have again gained relatively, the females are also absolutely much faster than are younger females.

Finally, a series of tests were run on flies 4 to 6 days old as follows. An initial test was run at the same time of day at which it had been the custom to remove newly hatched flies from their bottles. They were then tested at the same relative intervals as the newly hatched insects had been in the experiments described above. In this case ten males and ten females were used in each group, and the number of trials constituting a test was raised to 5. The results were as follows (table 7):

TABLE 7

Male indices

| 0 HRS. | 4 HRS. | 12 HRS. | 15 HRS. | 18 HRS. | 21 HRS. | 24 HRS. |
|--------|--------|---------|---------|---------|---------|---------|
| 83.6 | 86.5 | 88.5 | 86.6 | 86.8 | 96.6 | 98.8 |

Female indices

| | | | | | | |
|------|------|------|------|------|------|------|
| 96.8 | 98.3 | 97.6 | 97.1 | 96.8 | 96.1 | 99.0 |
|------|------|------|------|------|------|------|

Temperatures

| | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|
| 22° | 22° | 21° | 21° | 21° | 21° | 22° |
|-----|-----|-----|-----|-----|-----|-----|

The main feature of this series is that there is no drop occurring in the middle of the series, such as was the case with the young flies tested at similar intervals. It is thus made more likely that the falling off in question was due, as suggested, to the more rapid fatigue of newly hatched insects. Incidentally, it will be noted that the males are not far behind the females, and that they gain on them during the series.

In conclusion, it may be said that females are never twice as active as males. They are, however, somewhat more active, particularly when only 1 or 2 days old. As age advances the difference between the sexes decreases until in some cases at 8 or 10 days the males actually surpass the females. Moreover, instead of the maximum period of phototropic response occurring at 18 hours, it would seem rather that both males and females, if not too heavily fed, increase their response with age, reaching a maximum in the neighborhood of 4 or 5 days. After this point both sexes tend to become less active, the females more rapidly than the males. It may also be added that the young flies fatigue much more rapidly than do older insects.

EFFECTS OF OPERATIONS ON THE REACTIONS TO LIGHT

a. Removal of wings

The operation of removal of the wings was suggested by Dr. T. H. Morgan as a laboratory experiment for one of his classes. Mr. S. Safir was the first to try the experiment, and obtained the rather surprising result that flies so treated no longer showed any response to light. This effect was so unexpected that it was determined by the writer to investigate the matter as thoroughly as possible.

The first experiments performed in this connection were undertaken with a view to determining whether the insects would recover their normal response if kept a sufficient length of time after the operation. As these tests were made at the beginning of the work no apparatus was employed except the tube and light from the north window. One fly was tested at a time and its record calculated according to method I, for instance, the fly was placed alternately in the end of the tube toward the light and in the end away from the light, and the algebraic sum of the average of the two sets of records in inches crawled was taken as the index of the fly in question. Five groups of animals were tested, in which the number of insects varied from one to five. When there was more than one fly in a group, the index of the group as a whole was computed by adding algebraically

the indices of the individuals constituting the group. It is the indices thus obtained that are set down in table 8. From these results it would appear that there is a slight recovery of phototropism. Nevertheless, in view of what occurs in normal flies

TABLE 8¹

| GROUP | DAYS | | | | | | | | | |
|------------------|------|------|-----|------|-----|------|-----|------|-----|------|
| | 1 | | 2 | | 3 | | 4 | | 5 | |
| | tl. | fl. | tl. | fl. | tl. | fl. | tl. | fl. | tl. | fl. |
| I..... | | 24.0 | | 17.6 | | 9.3 | | 8.6 | | 4.3 |
| II..... | 00.7 | | | 15.3 | | 12.8 | 2.0 | | | 2.3 |
| III..... | | 10.0 | | 8.9 | | 17.3 | | 18.5 | | 9.9 |
| IV..... | | 4.3 | | 1.0 | | 00.7 | | 00.9 | | 3.0 |
| V..... | | 00.4 | | 00.3 | | 2.0 | | 4.7 | | 0.4 |
| Totals..... | 00.7 | 38.7 | | 43.1 | | 42.1 | 2.0 | 32.7 | | 19.9 |
| Average..... | 00.7 | 9.6 | | 8.6 | | 8.4 | 2.0 | 8.1 | | 3.9 |
| Differences..... | | 8.9 | | 8.6 | | 8.4 | | 6.1 | | 3.9 |

| GROUP | DAYS | | | | | | | | | |
|------------------|------|-----|------|-----|------|------|------|-----|------|------|
| | 6 | | 7 | | 8 | | 9 | | 10 | |
| | tl. | fl. | tl. | fl. | tl. | fl. | tl. | fl. | tl. | fl. |
| I..... | | 1.7 | 7.0 | | 15.0 | | 18.4 | | 10.0 | |
| II..... | 10.3 | | 3.6 | | 16.0 | | 4.3 | | 13.7 | |
| III..... | 3.9 | | | 4.2 | | 14.2 | 8.1 | | | 00.7 |
| IV..... | 10.3 | | | 2.9 | 5.7 | | 4.1 | | 18.6 | |
| V..... | | 4.4 | | 1.7 | 4.3 | | 3.6 | | | |
| Totals..... | 24.5 | 6.1 | 10.6 | 8.8 | 41.0 | 14.2 | 38.5 | | 42.3 | 00.7 |
| Average..... | 8.1 | 3.0 | 5.3 | 2.9 | 10.2 | 14.2 | 7.7 | | 14.1 | 00.7 |
| Differences..... | 5.1 | | 2.4 | | | 4.0 | 7.7 | | 13.4 | |

¹ No temperature was taken in these early experiments, but later tests made in the same room under similar conditions showed a variation of less than a degree during a period of two weeks.

tl. indicates excess of inches crawled toward light, resulting from the algebraic sum of indices in the group. fl. indicates excess of inches crawled away from the light, calculated in the same manner. Diff's. indicates the algebraic sum of the fl and tl averages.

TABLE 8—(Concluded)

| GROUP | DAYS | | | | | |
|------------------|-------|-----|------|-----|------|-----|
| | 11 | | 12 | | 13 | |
| | tl. | fl. | tl. | fl. | tl. | fl. |
| I..... | 113.3 | | 22.9 | | 27.3 | |
| II..... | 25.0 | | 22.6 | | | |
| III..... | | 4.3 | | | | |
| IV..... | | | | | | |
| V..... | | | | | | |
| Totals..... | 38.3 | 4.3 | 45.5 | | 27.3 | |
| Averages..... | 19.1 | 4.3 | 22.7 | | 27.3 | |
| Differences..... | 14.8 | | 22.7 | | 27.3 | |

with advancing age, it seems likely that this recovery represents nothing more than the usual increase. It should be noted that the usual index for unmutilated flies under this system of recording is from 30 to 36. This will appear in the next experiment.

In this experiment, also under method I, five groups of flies were used. In the first group the wings were not removed until the sixth day after hatching, while in the fifth group they were removed on the day of hatching. The results were the same in every case. The insects showed the usual positive reaction to light until the day the wings were removed. At this point the positive reaction disappeared, the insects being indifferent to light and remaining substantially so for the rest of the tests. This occurrence was perfectly regular and very striking. It will therefore suffice to give only a couple of illustrations. In Group I there was only one fly. On the day after hatching, a t. l. index of 30.4 was recorded. Its record was about this each day until the sixth, when the wings were removed. For the subsequent five tests its average was f. l. 1.7. On the sixth test it went up to t. l. 4.7, but on the seventh it dropped to t. l. 2 and on the eighth and last the record was f. l. 1. In Group IV there were five flies. On the day after hatching they averaged t. l. 33.4. The day following their wings were removed, one insect having been lost in the meantime. On this, the third day after

hatching, they now averaged f. l. 3.1. Thus, it does not appear that the age at which the wings are cut off has anything to do with the effect.

It should be added that the slight f. l. excesses recorded in the above experiments are probably not significant. From watching the actual behavior of the flies it did not appear to me that the operation did any more than to render them practically indifferent to light. Indeed, I have never observed a clearly negative reaction in *Drosophila*.

So far the apparent loss of phototropism might mean merely that the operation had made the insects inactive. However, since *Drosophila* is strongly negatively geotropic it was possible to use this reaction as a measure of general activity.³ For this purpose the system of testing several insects at a time, known as method II was used. The flies were introduced into the usual testing tube and given one trial for the reaction to light in the regular manner, except that no agitation was employed. Following this the tube was fastened in a vertical position with the flies at the bottom, and at a distance of 41 cms. from a 100 watt tungsten lamp hung so that its tip just touched the table. Three such tests were given, alternating with three light tests, and the indices for the two sets calculated as usual for the above method. The elimination of agitation in these tests was made necessary in order to make comparable the records of the flies with and without wings. When agitated the former move to-

³ Regarding the relative strength of the two stimuli, light and gravity, Cole decides in favor of the latter. He found that when flies were placed in a vertical cylinder illuminated from below the larger per cent went to the uppermost third. Carpenter, on the other hand, was able to attract the insects to the bottom of a similar cylinder without using as strong a light as did Cole. On account of the great variability of *Drosophila*, I suggest that this discrepancy may be due to the small number of flies used, Cole employing only twenty-one and Carpenter only six.

My own results are not strictly comparable with those of either of these authors, because I used a type of apparatus which did not directly oppose the two stimuli, but such evidence as I have agrees with that of Carpenter. Thus, a reference to any of the tables where the light and gravity indices of normal insects are recorded will show that the light index in any given case always exceeds that for gravity. In any event, the matter of which stimulus is the stronger is not one of any great significance.

ward the light both by crawling and flying, whereas the latter can only crawl. When not agitated, however, the response even of winged insects is almost purely a crawling reaction.

Three groups of flies containing 20 insects each were now selected at random from the stock bottles. They were placed in vials in the morning and tested according to the above plan in the afternoon. After this first test all the insects were etherized and the wings removed from the two groups which had made the best record. These groups will be designated as B and C. The following afternoon all three groups were tested again. Following are the records of Groups B and C before and after the wings were removed, and also the two records of the control Group A (table 9).

TABLE 9
Before removal. Temperature 24°

| A | | B | | C | |
|----------------------|---------|---------|---------|---------|---------|
| Gravity | Light | Gravity | Light | Gravity | Light |
| (First test) | | | | | |
| Males | Males | Males | Males | Males | Males |
| 29.1 | 57.6 | 28.3 | 75.0 | 21.6 | 65.0 |
| Females | Females | Females | Females | Females | Females |
| 31.6 | 81.6 | 45.0 | 90.8 | 32.5 | 85.0 |
| <i>After removal</i> | | | | | |
| (Second test) | | | | | |
| Males | Males | Males | Males | Males | Males |
| 50.0 | 86.6 | 35.0 | 5.8 | 39.1 | 20.8 |
| Females | Females | Females | Females | Females | Females |
| 41.6 | 90.8 | 30.0 | 7.5 | 25.0 | 37.5 |

As a further check, Group A, the control, was tested a third time 48 hours after the second test. The wings were then removed and eight hours later a fourth test was given (table 10). It is evident from this data that the removal of the wings affects the light reaction specifically, and does not merely reduce the activity of the insects.

The next point investigated was the effect of the removal of parts of these appendages. The first experiment was done un-

TABLE 10
Group A. Temperature 23°

| | GRAVITY | | LIGHT | |
|--------------------------------------|---------|---------|-------|---------|
| | Males | Females | Males | Females |
| Third test, before wing removal..... | 46.6 | 30.0 | 88.3 | 84.1 |
| Fourth test, after removal..... | 43.3 | 40.0 | 11.6 | 6.6 |

der method I, the age of the insects being unknown. The apparatus was exactly the same as that described in connection with the effect of removing the wings at different times after hatching. Five groups of flies were employed in the first series of tests. In the case of the first two groups only half of the wings were removed throughout the tests, while in the case of the last three groups one or two tests were given with half the wing removed, and then a second operation was performed in which three-fourths of the total wing was taken. Table 11 summarizes the results.

It appears that though the insects are very erratic and vary much from time to time, those animals which had had the wing completely removed, with one single exception, made lower records than any made by flies with only half of the wings removed. These experiments are unsatisfactory, however, in failing to show what, if any, effect the removal of half the wing has. Later on, therefore, another set of experiments was devised to answer this question. In this case method II was used with the improved apparatus. Likewise, the alternating gravity trials were introduced as a control. In short, the general method was precisely similar to that employed in the proof that wing removal has a specific effect on the reaction to light (table 9).

Four groups of flies were selected at random from the bottles and run through the tests. The flies were then etherized and treated in the following way. Group I, which contained nine males and ten females, had made the lowest record and was restored to the vials without operation. Group II, which contained ten males and eleven females, and had made next to the lowest record, had only the tip ends of the wings removed. Group III, which contained ten males and nine females and had the second best record, had one-half of the wings cut off, and

TABLE 11

| NUMBER OF FLIES | GROUP | TESTS | ONE-HALF WING REMOVED | | THREE-FOURTHS WING REMOVED | |
|--------------------|-------|-------|-----------------------|-------|-------------------------------|-------|
| | | | t. l. | f. l. | t. l. | f. l. |
| 6 | I | 1 | 144.4 | | | |
| 6 | I | 2 | 28.8 | | | |
| 6 | I | 3 | 129.1 | | | |
| 6 | I | 4 | 61.3 | | | |
| Average..... | | | 90.9 | | | |
| 6 | II | 1 | 57.0 | | | |
| 6 | II | 2 | 8.9 | | | |
| 6 | II | 3 | 52.3 | | | |
| Average..... | | | 39.4 | | | |
| 6 | III | 1 | 104.3 | | | |
| 6 | III | 2 | | | | 38.6 |
| 6 | III | 3 | | | 8.8 | |
| 6 | III | 4 | | | | 24.2 |
| Average..... | | | 104.3 | | 8.8 | 31.4 |
| 6 | IV | 1 | 69.0 | | | |
| 5 | IV | 2 | | | | 20.3 |
| 5 | IV | 3 | | | | 32.3 |
| Average..... | | | 69.0 | | | 26.3 |
| 6 | V | 1 | 48.1 | | | |
| 6 | V | 2 | 46.4 | | | |
| 6 | V | 3 | | | | 9.1 |
| 6 | V | 4 | | | | 10.2 |
| 4 | V | 5 | | | | 1.1 |
| Average..... | | | 47.2 | | | 6.8 |

Average for one-half wings.....t.l. 70.1

Average for three-fourths wings.....f.l. 12.7

Group IV, which had made the best record and contained ten males and six females had three-fourths of every wing removed. The next day all groups were retested with the following results (table 12):

TABLE 12

| | GROUP I. WINGS NOT CUT | | | | GROUP II. WINGS CUT ONE-FOURTH | | | |
|---------------------|---------------------------|--------|---------|--------|-----------------------------------|--------|---------|--------|
| | Light | | Gravity | | Light | | Gravity | |
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Before cutting. | 66.6 | 82.5 | 27.7 | 26.6 | 80.0 | 88.5 | 27.5 | 24.9 |
| Temperature..... | 23.5° | | | | 23.5° | | | |
| After cutting..... | 91.6 | 92.5 | 48.1 | 48.3 | 76.6 | 72.5 | 40.0 | 36.6 |
| Temperature..... | 24° | | | | 24° | | | |
| | GROUP III. WINGS CUT HALF | | | | GROUP IV. WINGS CUT THREE-FOURTHS | | | |
| | Light | | Gravity | | Light | | Gravity | |
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Before cutting..... | 80.0 | 95.3 | 41.6 | 42.5 | 90.8 | 95.8 | 40.8 | 38.3 |
| Temperature..... | 23.5° | | | | 23.5° | | | |
| After cutting..... | 19.1 | 21.2 | 65.0 | 49.0 | 25.8 | 26.3 | 61.6 | 49.9 |
| Temperature..... | 24° | | | | 24° | | | |

It is evident from these figures that with a single exception, such as the case of the groups in which three-fourths of the wing was removed, these results support the conclusion that the decrease in phototropism is directly proportional to the amount of the wings removed. Moreover, in considering this result and particularly the one exception, it must be remembered that the amount of wing cut off in each group was directly proportional to the height of the index originally scored by that group in the initial tests. Thus, the group having three-fourths of the wing removed was originally the fastest of all, and this may account for its still retaining enough speed to win out over the group with only one-half of the wing removed. This seems particularly probable when this experiment is considered together with the previous one. Taking the two together, I believe we are justified in the conclusion that at least roughly speaking the proportion between phototropism and wing length holds good.

b. Gluing the wings

Several attempts were made to glue the wings in such a way that though uninjured, the insect could not use them. These attempts were made fruitless, however, by the fact that a fly whose wings are stuck thoroughly enough so that they can not be freed, will spend all its time in an effort to do so, and will scarcely respond to any other stimulus during the process. This experiment, therefore, had to be given up.

There remained two other possibilities. First, the effect of operations as such could be determined, by operating on other parts of the insect. Secondly, the existence in this laboratory of mutations of all degrees of winglessness made it possible to discover the effect of the absence, or partial absence, of wings in *Drosophila* upon which no operation had been performed. The effects of other operations will be considered first.

c. Cutting off legs

For this purpose eleven males and ten females, newly hatched, were selected and kept in vials until five days old, this being the usual procedure when records comparable with those made by other groups were desired. Before placing in the vials each insect was operated on, and the tarsus and tibia of the middle pair of legs cut off. It was thought that removal of the middle pair in this manner would interfere least with the animal's balance and ability to crawl. On the fifth day these flies were tested with a resulting index of 53.1 for the males and of 86.3 for the females. Under similar conditions it will be recalled that a normal index would be approximately 95 and 97, though I have cases where it was considerably lower. Thus, though there may be a slight effect from this operation, it is obviously not very great. Furthermore, it must be remembered that however quickly and accurately these flies might orient, they were necessarily handicapped in their speed of movement except when they took to wing. Since this experiment was performed under agitation this was frequently the case. As a matter of fact, orientation and movement toward the light was perfectly constant

TABLE 13—*Concluded*
With antennae removed

| | TESTS | LIGHT | | GRAVITY | | | |
|------------------------------|-------|-----------------------|--------|----------|--------|------------|--------|
| | | Male | Female | Vertical | | Horizontal | |
| | | | | Male | Female | Male | Female |
| Set A..... | 1 | 91.0 | 66.5 | 0.5 | 1.0 | 6.0 | |
| | 2 | 86.0 | 81.5 | 11.0 | 6.0 | 27.5 | 6.0 |
| | 3 | | | 11.0 | | 17.0 | |
| Temperature..... | | 23° | | 23° | | | |
| Set B..... | 1 | 92.5 | 89.5 | 21.5 | 17.5 | 17.0 | 27.0 |
| | 1 | | | | | | |
| Temperature..... | | 24° | | 24° | | | |
| Set C..... | 1 | 77.5 | 100.0 | 13.0 | 15.0 | 10.5 | 12.0 |
| | 2 | 71.0 | 90.1 | 15.5 | 16.5 | 14.0 | 13.0 |
| Temperature (Group b)..... | | 23° | | 23° | | | |
| Set D..... | 1 | Also wings clipped | | 16.0 | | 13.5 | |
| | 2 | | | 12.1 | | 5.5 | |
| Temperature..... | | 23° | | | | | |
| Total..... | | 418.0 | 427.6 | 142.1 | 104.0 | 140.0 | 85.5 |
| Averages..... | | 83.6 | 85.5 | 15.7 | 17.3 | 15.5 | 17.1 |
| Vertical excess, male..... | | | | | | | 0.2 |
| Vertical excess, female..... | | | | | | | 0.2 |

these tests, each will have to be described separately. The results, however, will be found summarized in table 13.

In Set A, two groups of twenty flies were removed from the bottle shortly after hatching. One group was operated on at once, while the other was kept as a control. Five days later the group from which the antennae had been removed was tested for light reaction. Three hours later both groups were given a gravity test. In this case this test consisted of two series of five trials each. In the first series the tube was held vertically, and the index calculated as usual, the result being designated as the vertical index. The second series acted as a control, the tube

being placed horizontally and the result designated as the horizontal index. The difference between these indices may thus be taken as the index of the animal's reaction to gravity. After these tests the flies with the antennae removed were given fresh food and kept for 3 days. The light and gravity tests were then repeated. On the following day, for instance, 9 days after hatching, the males of this group were given one more gravity test. At this point the alternating system for the vertical and horizontal positions of the tube was introduced, and used in all the subsequent tests of this experiment.

In Set B three groups of flies were taken and from one group the antennae were removed. The other two were used as controls. Five days later one control group and the one from which the antennae had been cut were given the light and gravity tests as in Set A. The second control group was given only a gravity test.

In Set C two groups of twenty insects were taken and from one group the antennae were removed as usual. At 5 days the animals which had been operated on were tested for light and for gravity. The control was tested for gravity only. Three days later the former group was again subjected to both light and gravity tests.

Set D consisted of two groups of male flies only, each having been used previously in tests on the effect of wing removal. One group, which we will designate as a, had been used as a control and had not had the wings removed. In the other group, b, the wings had been cut off. This latter group was now etherized and the antennae as well as the wings were taken off. The control group was etherized at the same time but no operation performed. Four hours later a gravity test was given to each group, and 3 days later these tests were repeated.

From the results of this experiment it appears that there has possibly been a very slight reduction in phototropic response. However, it is certainly in no way comparable with the reduction which occurs regularly as the result of wing removal. Furthermore, a study of the light responses of normal insects contained in other tables, shows such variation that it is extremely doubtful

if the slight falling-off of some of the antennaeless groups in table 13 is of any significance at all. From this result, therefore, as well as that obtained by the removal of legs we are led to conclude that any operation as such is not sufficient to cause a loss of phototropism. Incidentally, however, a rather interesting result does appear here as to the specific effect of the removal of the antennae and reaction to gravity. From the small amount of data on hand, it appears that the loss of these organs greatly reduces a fly's negative geotropism. It also seems to produce a slight reduction in general activity. There are not, however, sufficient data collected on these particular points to do more than suggest a line for further investigation.

EXPERIMENTS ON MUTANT WING-CHARACTERS

We are now in a position to attempt the second method of analyzing our problem by testing the various sorts of wing mutants which have arisen in this laboratory. These mutants vary all the way from vestigial, in which the wings are mere stubs to curled, in which the wings though of normal length are turned upward at the end and are not very effective in flying. There are many other variants between these two, one of which is designated as strap. Strap has wings almost as long as normal, but they are narrow, often cleft at the end, held off from the body at a peculiar angle and are useless for flight. These three mutants therefore were selected as bearing the closest approximation to insects with one-fourth, one-half and three fourths of the wing removed. The flies in question are represented in figure 2. A normal insect is also included for the sake of comparison.

The tests on the above mutants have all been made according to the plan already outlined in one of the experiments for testing the effect of the removal of wings on light and gravity (p. 69). In brief, three tests were made for light, alternating with three tests for gravity, with the tube in the vertical position only. No agitation was used during any of these tests. Only one new point needs to be mentioned and that is in regard to an improvement in the apparatus. Under the former system of testing for geotropism the lamp whose rays struck the tube at

right angles was nevertheless near its foot. It was recognized that this method was unreliable when we attempted to compare the gravity reaction of flies which were phototropic with that of those which were not. Thus, two sets of flies, one phototropic and the other not, but possessing an equal amount of

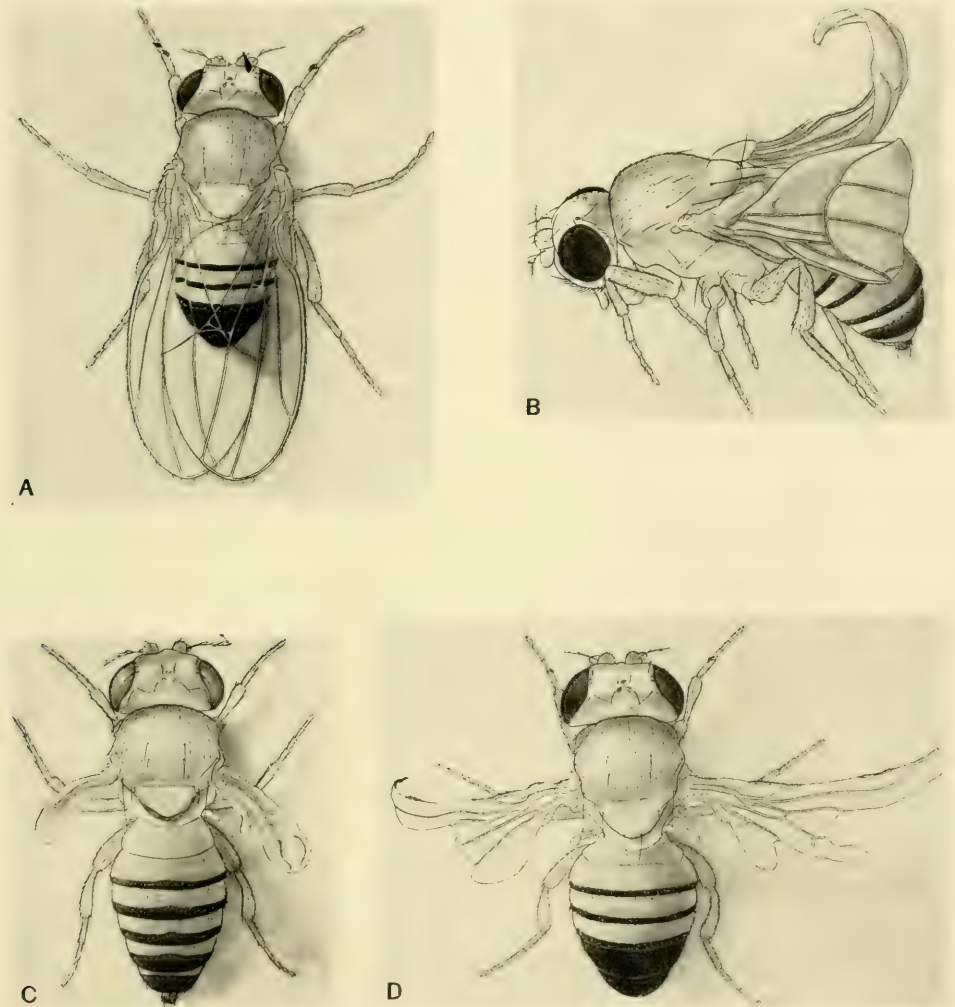


Fig. 2. A, Normal insect. B, Specimen of 'curled' stock. C, Specimen of 'vestigial' stock. D, Specimen of 'strap' stock.

negative geotropism would show the latter more negatively geotropic than the former. This would result from the fact that though equally impelled to move upward, the phototropic animals would be constantly handicapped by the attraction of the light from below. In order to remedy this difficulty, therefore, three lamps of the same candle power were arranged in a vertical line, so that one came opposite the foot of the tube, one opposite the center, and one opposite the top. The end of each bulb was a distance of 41 cm. from the tube. The latter, moreover, was now held in its vertical position by a wire support, so that there was no danger of its wobbling. With these improvements, the following experiments were undertaken.

In the first place, it was decided to run a test on some wild flies in order to get some data which should be comparable with that obtained by the same system for the mutants. Also in making these tests it was decided incidentally to run a few checks on the effect of wing removal, in order to make sure that the former tests were not invalidated by the position of the single lamp. For this purpose three groups of insects, each containing ten males and ten females, were selected, and kept in the usual manner until 5 days old. They were then tested as described above. After the test, the males in the groups which we shall designate as A and B were etherized and in the case of group B the wings were removed. Eight hours later both groups were retested. The control males in Group A were now also operated on, and tested for the third time 10 hours later. In the case of the females, Group A was operated on after the first test and Group B kept as a control. Twenty-four hours later both these female groups were tested again. Group B was now operated on and tested after eight hours. The results of these tests are summarized below (table 14).

This experiment confirms the conclusions already set forth regarding the effect of wing removal. In other words, the rearrangement of the lights has produced no significant effect. The only thing of note in the record is the extremely high gravity index registered by the males of Group B after being operated on. To determine whether this is of any significance or not will require

TABLE 14

| | TESTS | BEFORE WING REMOVAL | | | | AFTER WING REMOVAL | | | |
|------------------|-------|---------------------|--------|--------------------|--------|--------------------|--------|--------------------|--------|
| | | Light | | Gravity (vertical) | | Light | | Gravity (vertical) | |
| | | Male | Female | Male | Female | Male | Female | Male | Female |
| Group A..... | 1 | 96.6 | 98.3 | 68.3 | 77.5 | | | | |
| | 2 | 96.6 | | 66.6 | | | 41.6 | | 50.0 |
| | 3 | | | | | 5.8 | | 45.0 | |
| Temperature..... | | 23.5° | | | | 23.5° | | | |
| Group B..... | 1 | 98.3 | 99.1 | 67.5 | 64.1 | | | | |
| | 2 | | 97.5 | | 55.0 | 52.5 | | 91.6 | |
| | 3 | | | | | 35.8 | 10.1 | 80.0 | 42.5 |
| Temperature..... | | 23.5° | | | | 23.5° | | | |
| Group C..... | 1 | 84.1 | 94.1 | 35.0 | 25.8 | | | | |
| Temperature..... | | 24° | | | | 24° | | | |
| Totals..... | | 375.6 | 389.0 | 237.4 | 222.4 | 94.1 | 51.7 | 216.6 | 92.5 |
| Averages..... | | 93.9 | 97.2 | 59.3 | 55.6 | 31.3 | 25.8 | 72.2 | 46.2 |

more data. It is true that a somewhat similar tendency is manifest among the males in table 9, but the poor light arrangement in the earlier experiments makes the records of doubtful validity on this particular point.

Let us now consider the reactions of vestigial flies. Three groups of insects, half male and half female, were kept for the usual length of time after hatching, and then subjected to the test just described for wild flies. The only difference was that in this case the single lamp was used in the gravity trials. As will appear from the results, however, this feature was of no consequence in this instance because the insects were only very slightly phototropic. Also since the wings were already only stubs, nothing was cut off. Table 15 summarizes the results.

Strap stock was next tested. Three groups, constituted as in vestigial and wild were kept as usual till 5 days old. The only irregularity in this connection was the use of only nine instead of ten females in Group A. As to the apparatus, the single

TABLE 15
Temperature 24°

| | LIGHT | | GRAVITY (VERTICAL) | |
|--------------|-------|--------|--------------------|--------|
| | Male | Female | Male | Female |
| Group A..... | 20.0 | 7.5 | 31.6 | 24.1 |
| Group B..... | 13.3 | 10.0 | 24.1 | 20.8 |
| Group C..... | 10.0 | 11.6 | 42.5 | 35.0 |
| Totals..... | 43.3 | 29.1 | 98.2 | 79.9 |
| Average..... | 14.4 | 9.7 | 32.7 | 26.6 |

lamp in the gravity trials was used in the first tests of the males in Groups A and B. After the first test the males of Group A were kept as a control, while those of Group B suffered the removal of the rather poorly developed wings which they possessed. Both sets were retested 8 hours later. Table 16 gives the results:

The results from this experiment are enough to suggest strongly the slight increase in phototropic response which might be expected to distinguish these flies from the vestigials. The most

TABLE 16

| | TESTS | BEFORE WING REMOVAL | | | | AFTER WING REMOVAL | | | |
|-----------------|-------|---------------------|--------|--------------------|--------|--------------------|--------|--------------------|--------|
| | | Light | | Gravity (vertical) | | Light | | Gravity (vertical) | |
| | | Male | Female | Male | Female | Male | Female | Male | Female |
| Temperature 24° | | | | | | | | | |
| Group A..... | 1 | 32.5 | 17.6 | 45.0 | 30.8 | | | | |
| | 2 | 62.0 | | 71.2 | | | | | |
| Group B..... | 1 | 42.5 | | 45.0 | | 63.8 | | 68.4 | |
| | 2 | | | | | | | | |
| Temperature 23° | | | | | | | | | |
| Group C..... | 1 | 10.0 | 24.1 | 41.6 | 48.3 | | | | |
| Total..... | | 147.0 | 41.7 | 202.8 | 79.1 | 63.8 | | 68.4 | |
| Averages..... | | 36.7 | 20.8 | 50.7 | 39.5 | 63.8 | | 68.4 | |

striking point which distinguishes these data, however, is the failure of wing removal to affect light reaction in Group B. Indeed, the test after the operation shows an actual increase in the reaction to light. In view of the rather low light indices in all the flies of this variety, I am inclined to explain this as follows: Even normal insects whose wings have been removed, vary a good deal from time to time in their degree of response to light. Also the general variability is such that a fly with three-fourths of its wings gone will not always show a lower response than one with only one-half gone. I therefore suggest that since these flies already have a low index on account of their imperfect wings, the removal of the remainder of the wing might not have sufficient additional effect to counterbalance some unknown change in the physiological condition of the animal. This statement is partially borne out by the fact that the males in Group A which were not operated on, also showed a markedly higher index for both light and gravity in the second test. Further experiments now under way will serve to show whether this is the true explanation or not. If it is not, we should have to accept the rather astonishing hypothesis that a fly with short wings and a low index to begin with, actually has its phototropism increased instead of diminished by the removal of such wings as it has.

Finally, we have to consider the reaction of the flies designated as curled. As usual, three groups of insects, constituted as in the previous tests of this series, were tested when 5 days old. As this particular group was really the first of the series the use of the single lamp in the gravity test was still customary. The change to the new system was, indeed, made during the work on this group, which accounts for the fact that only the males in Group A were subjected to the improved treatment. That this feature was really of no great significance, at least in the case of these flies can be told by comparison of Group A's record with those of the others. The males of both Groups A and B had their wings removed after the first test and were retested eight hours later. Group C males were not operated on, but were retested after the same interval of time as a control.

In both Groups A and B one fly was lost before the re-test. Table 17 shows the results.

For the sake of comparison the average indices for light and gravity obtained from the above experiments on wild, vestigial,

TABLE 17
Temperature 24°

| | TESTS | BEFORE WING REMOVAL | | | | AFTER WING REMOVAL | | | |
|---------------|-------|---------------------|--------|--------------------|--------|--------------------|--------|--------------------|--------|
| | | Light | | Gravity (vertical) | | Light | | Gravity (vertical) | |
| | | Male | Female | Male | Female | Male | Female | Male | Female |
| Group A..... | 1 | 83.3 | 34.1 | 86.6 | 40.0 | | | | |
| | 2 | | | | | 55.5 | | 83.3 | |
| Group B..... | 1 | 83.3 | 51.6 | 80.8 | 45.0 | | | | |
| | 2 | | | | | 69.4 | | 74.0 | |
| Group C..... | 1 | 75.8 | 48.3 | 81.6 | 49.1 | | | | |
| | 2 | 83.3 | | 85.0 | | | | | |
| Total..... | | 325.7 | 134.0 | 334.0 | 134.1 | 124.9 | | 157.3 | |
| Averages..... | | 81.4 | 44.6 | 83.5 | 44.7 | 62.4 | | 78.6 | |

strap and curled flies have been brought together in table 18. From this table it is evident that there is a steady drop in phototropic response which in a rough way is directly proportional to the lack of development of the wings.

The curious effect of defective wings upon light reaction as indicated by the above experiments made it desirable to examine

TABLE 18
Temperature 23°-24°

| AVERAGES FOR | BEFORE WING REMOVAL | | | | AFTER WING REMOVAL | | | |
|----------------|---------------------|--------|---------|--------|--------------------|--------|---------|--------|
| | Light | | Gravity | | Light | | Gravity | |
| | Male | Female | Male | Female | Male | Female | Male | Female |
| Wild..... | 93.9 | 97.2 | 59.3 | 55.6 | 31.3 | 25.8 | 72.2 | 46.2 |
| Curled..... | 81.4 | 44.6 | 83.5 | 44.7 | 62.4 | | 78.6 | |
| Strap..... | 36.7 | 20.8 | 50.7 | 39.5 | 63.8 | | 68.4 | |
| Vestigial..... | 14.4 | 9.7 | 32.7 | 26.6 | | | | |

these organs carefully in order to see if they might possibly contain any sort of light receiving structures. A number of minute organs were found (fig. 3). Except for the seven larger ones which occur well out on the veins, the majority are arranged in groups near the base. They have in fact very much the same arrangement and appearance as have the so called olfactory organs described by McIndoo for the honey bee ('14).

In order to discover whether these organs have anything to do with the reaction to light three groups of twenty flies each were selected and kept for the usual 5 days. In one group the

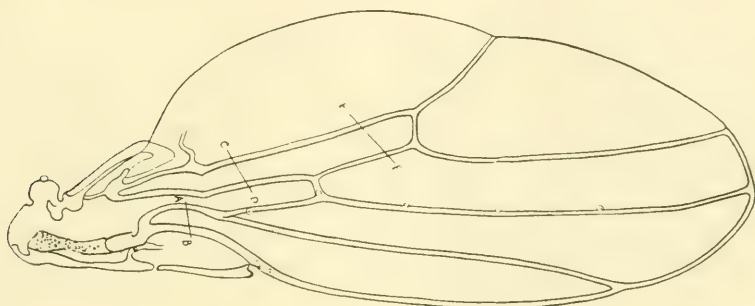


Fig. 3. *AB*, Line of cut made to isolate the larger sense organs. *EF*, *CD*, Line of cuts made as a control, on veins which contained no sense organs.

vein upon which occurred the largest number of organs was cut as shown by the line *AB*. In the other group the veins were cut along the lines *CD* and *EF*, while in the third group no operation was performed, though the flies were etherized as in the first two cases. Table 19 gives the result.

These operations were performed on the assumption that if the structures on the veins were light receiving organs, the nervous connection for such organs must pass along these veins. If such were the case, then the insects which had those veins cut on which some of the organs occurred, should have been most affected by the operation. As a matter of fact, however, flies in which the veins were cut which contained no sense organs were as much affected as the others. Furthermore, it should be noted

TABLE 19

| | VEIN CUT | | | | NO OPERATION | |
|-----------------------|----------|---------|-----------|---------|--------------|---------|
| | A B. | | C D. E F. | | | |
| | Males | Females | Males | Females | Males | Females |
| Before operation..... | 100.0 | 99.1 | 93.3 | 95.8 | 93.3 | 80.8 |
| After operation..... | 43.1 | 41.6 | 40.8 | 46.6 | 92.5 | 83.3 |

that in neither case was that part of the wing injured where the chief groups of organs occur. These points make it pretty clear that the effect produced on light reaction due to injuring the fly's wing is not the result of injury to these particular organs.

The fact that the stimulus for the light reaction is received in large part at least through the eyes and not through the wings or other organs is attested by the following experiment. In one of the mutant stocks known as eyeless, the compound eyes are very poorly developed, and in the case of many of the females are entirely lacking. Two groups of twenty flies each were, therefore, selected, one group containing only the individuals with the best developed eyes, and the other only those males with poorly developed eyes, and those females with no eyes at all. At the age of 5 days a light and gravity test was given these insects with the following results (table 20).

TABLE 20

| EYES PRESENT | | | | EYES POORLY DEVELOPED OR LACKING | | | |
|--------------|--------|---------|--------|----------------------------------|--------|---------|--------|
| Light | | Gravity | | Light | | Gravity | |
| Male | Female | Male | Female | Male | Female | Male | Female |
| 97.5 | 65.8 | 65.8 | 46.6 | 74.1 | 25.0 | 70.8 | 39.1 |

The data seems to indicate that at least a large share of the stimulus causing the light reaction is received through the compound eyes, since when these are undeveloped the response is greatly reduced, whereas that for gravity remains approximately the same.

We may now summarize the work which has been done on the relation of the fly's wing to its phototropic response as follows.

If the wings of *Drosophila* are removed, the insect's response to light is greatly reduced. Furthermore, if they are partially removed, the reduction in response is roughly proportional to the amount taken off. That such a reduction is really due to a loss of phototropism and not to a general decrease in activity is proven by the fact that the insect's response to the stimulus of gravity is reduced, very slightly, if at all. It now remained to show that the effect was directly due to the loss of the wings and not to the operation in itself. This has been accomplished first by performing other than wing operations and noting their effect and, secondly, by using breeds of insects which are hatched with imperfect wings. The operations performed involved the removal of legs and antennae. However, except in so far as general speed of locomotion was affected by the former operation, it could not be concluded that such injuries specifically affected the response to light. One incidental suggestion arising from these operations, however, is to the effect that removal of the antennae may materially affect the reaction to gravity. There is no obvious explanation for this, since Cole⁴ has shown that the stimulus of gravity is probably received through the leg muscles. The second method, namely, the use of vestigial, strap and curled wing flies gave results which still further bear out the hypothesis that it is the condition of the insect's wing as such that in some way directly affects the response to light. The possibility that sense organs on the wings were responsible for this peculiar result was tested by injuring the wing so as to break the nerve connection with some of these sense organs. It was found, however, that these organs had nothing to do with the response to light. That the stimulus for this response is received chiefly through the compound eyes was proved by testing eyeless stock containing individuals with and without these organs. Finally, the notion that the effect may be due to a variation in the weight of the wing is made very improbable by the fact that the wings of curled insects, though deformed, are apparently just as large as those of normals.

⁴ W. H. Cole, The reactions of *Drosophila ampelophela* Loew to gravity and air currents. *Jour. Animal Behavior*, Jan., Feb. 1917.

INHERITANCE OF PHOTOTROPISM IN DROSOPHILA

In the Biological Bulletin, 1911, Dr. Fernandus Payne gives the results of some phototropic tests made upon *Drosophila* which had been bred for 69 generations in the dark. In the course of the work he discovered, as I have done, that there was great variation among individuals and he therefore made an attempt to test the inheritance of the reaction. He was unable, however, to obtain any significant results and this, so far as I know, is the only effort to study the inheritance of this reaction in *Drosophila* that has ever been made.

It was with great interest, therefore, that I discovered that a certain stock of flies in this laboratory showed very little response to light. This stock was a combination of three separate mutants which were being carried along together for the sake of convenience. It is known as eosin, tan, vermillion. Eosin and vermillion are eye colors while tan refers to a slightly tan tinge to the body and a clear tan in the antennae. The antennae of the wild flies are gray. At first thought, of course, it appeared likely that the peculiar reaction to light was due to the light eye color. Stock in which these eye colors occurred without the tan were therefore secured and tested. Neither of these eye colors, however, were any less phototropic than normal. It is unnecessary to give the figures for the tests here, since experiments performed later in connection with colored lights amply demonstrate the phototropism of these breeds. The fact still remained that the peculiar reaction of eosin and vermillion might be due to a combination in one fly of the factors producing these characters. As tan had arisen as a mutation in eosin vermillion stock, the only way to test this was to get the tan separated from the two eye colors. By a suitable series of crosses this was ultimately accomplished. It was then possible to test tan by itself. The test immediately showed that either the factor for tan itself or some other factor very closely linked with tan, was responsible for the peculiar light reaction. Whenever an insect was homozygous for tan it failed to react to light. It may be stated at this point that tan is a recessive sex linked character. That is, the daughters inherit the factor for the character from

their fathers, but do not show the character, while sons inherit the factor from their mothers and do show the character.⁵

The independence of the light reaction from the color as such may be realized from the following facts. Tan, like most other characters, varies about a mode. Furthermore, this particular character is so delicate, that at the extreme of variation toward the normal color it is quite impossible to distinguish the individual possessing it from wild stock. This being the case, it sometimes happened that an insect whose genetic constitution was really tan would be accidentally mixed with flies which were supposed to be normal and *vice versa*. For example, one case occurred of a male fly which was apparently tan. Its light test, however, proved to be that of a normal. To test it, therefore, it was bred to a tan female. If the father were really tan, as it appeared, its daughters would all be tan. If, however, it were really normal as its light reaction indicated, then all its daughters would be normal. The latter turned out to be the case. The daughters were normal both in appearance and in light reaction. Later a case arose where several normal appearing females reacted as though tan. They were bred to a normal male. If they had been normal then all of the offspring should have been normal; if they were heterozygous then all the females would have been normal but half the males tan. What happened, however, was that all the females were normal, since tan is recessive, but all the males were tan. This proved that the females were really all homozygous tans as they had indicated by their light reaction, though their appearance had belied the fact. Thus it developed that light reaction was a surer test for the character of tan than was the color itself.

It remains merely to give a table showing the records of three groups of twenty tan flies each. They were tested according to the most recent system of light and gravity tests (table 21).

Table 21 offers conclusive evidence that the failure of tan flies to respond to light is not due to any general inactivity. This is

⁵ For a full discussion of Mendelism in *Drosophila*, see the Mechanism of Mendelian Heredity by Morgan, Sturtevant, Muller and Bridges.

TABLE 21

| | LIGHT | | GRAVITY (VERTICAL) | |
|-----------------|-------|--------|--------------------|--------|
| | Male | Female | Male | Female |
| Temperature 24° | | | | |
| Group A..... | 16.6 | 7.5 | 53.3 | 41.6 |
| Temperature 23° | | | | |
| Group B..... | 7.5 | 5.8 | 78.3 | 44.1 |
| Group C..... | 18.3 | 20.8 | 81.6 | 70.8 |
| Totals..... | 42.4 | 34.1 | 213.2 | 156.5 |
| Averages..... | 14.1 | 11.3 | 71.0 | 52.1 |

further borne out by observation of the insects. They are fully as active as are those from normal stock.

Finally, a number of normal, white and vermillion eyed and tan flies have been sectioned and examined, both with and without staining. So far, no histological abnormality has been discovered in the eyes of the tan insects, to account for their peculiar lack of response to light.

EFFECT OF COLORED LIGHTS ON NORMAL AND MUTANT EYE COLORS

A very considerable amount of work has been done by various investigators upon the effects of different wave lengths on organisms which respond either positively or negatively to light. Though it has generally been found that animals as well as plants respond more readily to the more refrangible rays of the spectrum, such is by no means invariably the case. In *Daphnia*, for instance, Lubbock (Journ. of the Linnean Society, 1881) and others have found that the green and yellow rays are more effective than any of the others, including the blue and violet. Also in the case of simpler organisms, it has been shown by Engelmann (Mast's account, Mast "Light and the Behavior of the Lower Organisms") that *Bacterium photometricum* tends to form aggregations in the infra red.

The general apparatus used in this work has already been described. The composition of the liquids used, together with the

wave lengths transmitted by each, were as follows (table 22). The spectrum tests were made often enough to make sure that no fading of the colors was taking place.

TABLE 22

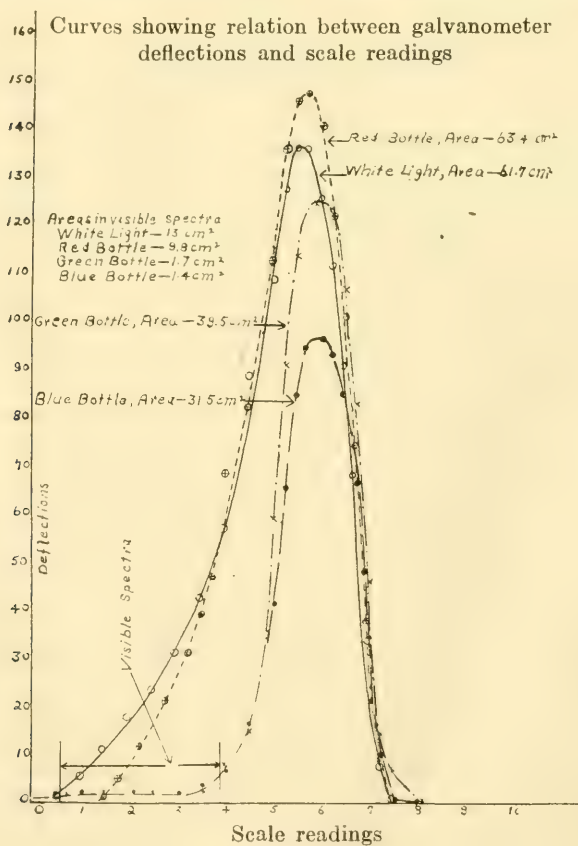
| | FORMULA | WAVE LENGTHS |
|-------------|--|---|
| Violet..... | Water.....285.0 cc. Ammonia..... 15.0 cc. Copper sulphate (filter)..... 7.5 grams | 5380 A° (green)-4240 A° (violet) Green strong at 4950 A° Violet strong at 4510 A° Blue, weak |
| Green..... | Water.....300.0 cc. Licht grün..... 0.03 grams Naphthol yellow... 0.25 grams Naphthol green.... 0.03 gram | 5660 A°-5050 A° Strongest at 5320 A° |
| Red..... | Water.....300.0 cc. Ponceau Red..... 3.0 grams | 7200 A°-6325 A° Strongest at 6570 A° |

The above formulae were only selected after a long series of experiments, and are for the most part modifications of formulae contained in the "Methods of Studying Vision in Animals" by R. M. Yerkes and John B. Watson, Behavior Monographs, 1911. The red and the green are very satisfactory for colors obtained by ray filters, while the so-called violet is evidently not so good. It is, as a matter of fact, continuous from green to violet. The blue, however, is very weak, the green moderate and the violet band very strong and wide. The results of the experiments show that it is probably not the green to which the effectiveness of this filter is due, and since the blue band is so slight, the probabilities are that violet is the effective stimulus. It is practically impossible to get a strictly violet filter. We find, however, that blue is obtainable, and it is intended to use such a filter in analyzing our results further at the earliest opportunity.

Besides the wave lengths, the relative energy transmitted by the filters was also measured by means of a thermocouple, using the same source of light employed during the experiments. The results are indicated in graph 4. From this it appears that if the energy transmitted by the colorless flasks be represented by 100

per cent, then the red flask transmits 103 per cent, the green 64 per cent and the violet 51 per cent. The fact that the red flask actually transmits more than the white is explained by the fact that the layer of clear water was slightly thicker than the red solution. It will be noted, however, that in the visible spectrum the red is somewhat less than the white, while the green and the violet are approximately equal.

The first colored light experiments performed were undertaken in the Zoological Laboratory of Western Reserve University, Cleveland, Ohio, during the summer of 1916. After some preliminary experimentation it was decided to make use of



Graph 4

method II.⁶ Four sets of flies were employed, each set consisting of six males and six females, the sexes being tested separately as usual. Every test consisted of three trials which were averaged to obtain the index for that test. Each of the four sets of flies was tested once for each of the three colors, the successive tests coming at intervals of 2 hours. For each set, however, the arrangement of the colors in the series was varied. Thus for set A it was red, green, violet; for set B, red, violet, green; for set C, violet, green; red; and for set D, green, red, violet. The results of these tests when averaged together for the four groups were as follows: males—violet, 64, red, 29.8, green, 24.5; females—violet, 81.8, red, 64.6, green, 57.2. In every one of the four sets violet was first in each complete test for males and females. As between green and red, red won in three out of the four sets for both males and females. Thus it would appear from this experiment that the colors are effective in the order—violet, red, green.

A further and better test was later made according to the following method. Two groups of insects each consisting of ten males and ten females, were picked out and designated as Group A and Group B. Each group was now tested six times at two hour intervals, with three trials to a test. In this case, however, the three trials constituting a test were not all of one color. Instead, there was a single trial for each of the three colors in every test. Furthermore, in each of the six tests the arrangement of the colors was altered according to a set plan. Group B was treated in exactly the same manner, except that the sequence of the color arrangements for each test in the series was reversed. Thus for test one the Group A arrangement was V, G, R; for test 2 V, R, G; for test 3 R, G, V; for test 4 R, V, G; for test 5 G, V, R; and for test 6 G, R, V. For Group B, the series began G, R, V and ended with V, G, R. At the end of the tests the indices for all the trials of a given color were averaged together in Group A and Group B. Finally the averages thus obtained for Group A and for Group B were averaged.

⁶ The tube in this experiment was only divided into four sections instead of the usual five.

Furthermore a record was kept in such a way that it was possible to see in how many trials a given color came out first, second, or third. It is evident that in this scheme, since every color was used in every test, the effect of previous tests would not change the relative value for any color in any given test. On the other hand the possible effect of the arrangement of colors in any given test is overcome by altering the arrangement every test. Finally, the method of recording gives not only the average of all the trials, but an analysis of individual trials. It, therefore, seems that a tolerably clear-cut result obtained in this way may reasonably be supposed of some significance.

This method was now applied in testing a series of mutant eye colors as well as the normal stock. The eye colors were as follows: white, an eye entirely lacking in pigment; tinged, almost white, but containing the lightest shade of red; eosin, a reddish yellow somewhat darker in the females; vermilion, a very good sample of this color; normal; and sepia. The last named color is virtually maroon on hatching, but grows darker with age until at five days it is practically black. The results from the tests on these stocks are summarized in tables 23 to 28. Graph 5 is based on the results for each eye color as indicated in groups A and B combined. Since there is no apparent sex differentiation as regards reaction to varied wave lengths, the graph has been constructed from the average male and female indices in every case.

It is evident from these tables that in the case of all colors lighter than normal, the general tendency is for the order of effectiveness to be violet, green, red. There are, however, three exceptions. First in group A of white eyed males, the red is ahead of the green both in the average index and in the number of tests in which this occurs. This case is more than offset, however, by group B, so that in the average of the two the order of colors is as stated above. It should be mentioned, moreover, that white eyed insects are extremely erratic even for *Drosophila*. It is quite usual for them after making a few consistent trips up the tube to become very much excited and to simply buzz about convulsively. The second exception is that

TABLE 23
White eyed flies
Group A

| SEX | | V | G | R | AVERAGE | | | |
|-----------|-----|----|----|---|---------------------------------------|--------|-------|------|
| | | | | | Sex | Violet | Green | Red |
| Male..... | 1st | 3 | 1 | 2 | Male..... | 72.9 | 49.9 | 52.5 |
| | 2d | 3 | 2 | 1 | Female... | 48.7 | 38.7 | 29.1 |
| | 3d | 0 | 3 | 3 | | | | |
| Female.. | 1st | 3 | 1 | 1 | Female { a. Tied once b. Tied once | | | |
| | 2d | 3a | 4b | 0 | | | | |
| | 3d | 0 | 1 | 5 | | | | |

Group B

| | | | | | | | | |
|-----------|-----|---|----|----|---|------|------|------|
| Male..... | 1st | 6 | 0 | 0 | Male | 61.3 | 28.2 | 15.1 |
| | 2d | 0 | 4 | 0 | Female... | 64.7 | 47.1 | 43.2 |
| | 3d | 0 | 2a | 6b | | | | |
| Female.. | 1st | 5 | 0 | 1 | Male { a. Tied twice. b. Tied twice Female { a. Tied once b. Tied once | | | |
| | 2d | 0 | 4 | 1 | | | | |
| | 3d | 1 | 2a | 4b | | | | |

Groups A and B combined

| | | | | | | | | |
|-----------|-----|----|----|----|--|------|------|------|
| Male..... | 1st | 9 | 1 | 2 | Male..... | 67.1 | 39.0 | 33.8 |
| | 2d | 3 | 6 | 1 | Female... | 56.7 | 42.9 | 36.1 |
| | 3d | 0 | 5a | 9b | | | | |
| Female.. | 1st | 8 | 1 | 2 | Male { a. Tied twice b. Tied twice Female { a. Tied once b. Tied once c. Tied once d. Tied once | | | |
| | 2d | 3a | 8b | 1 | | | | |
| | 3d | 1 | 3c | 9d | | | | |

of group A tinged females. Here the average green index very slightly exceeds the violet. The table indicating the number of times each color won, however, shows that from this standpoint violet is still well in the lead. The third exception is found in group B vermilion females where red very slightly exceeds green both in the average index and in the number of times which it was ahead. There is no special explanation for this except the fact that the eye color is approaching that of normal. In any case group A more than overbalances it.

TABLE 24

Tinged eyed flies

Group A

| SEX | | V | G | R | AVERAGE | | | |
|-----------|-----|----|----|----|--|--------|-------|------|
| | | | | | Sex | Violet | Green | Red |
| Male..... | 1st | 6 | 0 | 0 | Male..... | 100.0 | 100.0 | 79.1 |
| | 2d | 0 | 6 | 0 | Female... | 98.3 | 99.1 | 97.0 |
| | 3d | 0 | 0 | 6 | | | | |
| Female.. | 1st | 4 | 1 | 0 | Female { a. Tied twice b. Tied twice c. Tied three | | | |
| | 2d | 0 | 2 | 1 | | | | |
| | 3d | 2a | 3b | 5c | | | | |

Group B

| | | | | | | | | |
|-----------|-----|----|----|----|--|-------|------|------|
| Male..... | 1st | 4 | 0 | 0 | Male..... | 100.0 | 98.7 | 70.1 |
| | 2d | 2a | 6b | 0 | Female... | 100.0 | 97.9 | 97.5 |
| | 3d | 1 | 0 | 6 | | | | |
| Female.. | 1st | 6 | 0 | 0 | Male { a. Tied twice b. Tied twice Female { a. Tied three b. Tied three | | | |
| | 2d | 0 | 2 | 1 | | | | |
| | 3d | 0 | 4a | 5b | | | | |

Groups A and B combined

| | | | | | | | | |
|-----------|-----|----|-----|-----|--|-------|------|------|
| Male..... | 1st | 10 | 0 | 0 | Male..... | 100.0 | 99.3 | 74.6 |
| | 2a | 2d | 12b | 0 | Female... | 99.1 | 98.5 | 97.2 |
| | 3d | 0 | 0 | 12 | | | | |
| Female.. | 1st | 10 | 1 | 0 | Male { a. Tied twice b. Tied twice Female { a. Tied twice b. Tied five c. Tied six | | | |
| | 2d | 0 | 4 | 2 | | | | |
| | 3d | 2a | 7b | 10c | | | | |

Turning now to the results obtained from the tests on normal and sepia, we find that the early records for normal made during the summer of 1916 have been confirmed. The order of effectiveness is not violet, green, red, but violet, red, green. There is one exception to this in the group A males where the green slightly exceeds the red. Finally in the case of sepia, there is the same reversal of the relative effectiveness of red and green. This instance, however, is more clear-cut than is the case with normal, for with sepia the red exceeds the green in all respects with absolutely no exceptions.

TABLE 25
Eosin eyed flies
 Group A

| SEX | | V | G | R | AVERAGE | | | |
|-----------|-----|----|----|---|--|--------|-------|------|
| | | | | | Sex | Violet | Green | Red |
| Male..... | 1st | 4 | 1 | 0 | Male..... | 93.5 | 87.9 | 70.0 |
| | 2d | 2a | 5b | 0 | Female... | 89.5 | 85.7 | 70.0 |
| | 3d | 0 | 0 | 6 | | | | |
| Female.. | 1st | 5 | 0 | 0 | Male { a. Tied once b. Tied once Female { a. Tied once b. Tied once | | | |
| | 2d | 1a | 6b | 0 | | | | |
| | 3d | 0 | 0 | 6 | | | | |

Group B

| | | | | | | | | |
|-----------|-----|----|----|---|---------------------------------------|-------|------|------|
| Male..... | 1st | 4 | 0 | 0 | Male..... | 100.0 | 94.8 | 71.8 |
| | 2d | 2a | 6b | 0 | Female... | 96.2 | 92.0 | 70.8 |
| | 3d | 0 | 0 | 6 | | | | |
| Female.. | 1st | 5 | 1 | 0 | Male { a. Tied twice b. Tied twice | | | |
| | 2d | 1 | 5 | 0 | | | | |
| | 3rd | 0 | 0 | 6 | | | | |

Groups A and B combined

| | | | | | | | | |
|-----------|-----|----|-----|----|--|------|------|------|
| Male..... | 1sr | 8 | 1 | 0 | Male..... | 96.7 | 91.3 | 70.9 |
| | 2d | 4a | 11b | 0 | Female... | 92.8 | 88.8 | 70.4 |
| | 3d | 0 | 0 | 12 | | | | |
| Female.. | 1st | 10 | 1 | 0 | Male { a. Tied three b. Tied three Female { a. Tied once b. Tied once | | | |
| | 2d | 2a | 11b | 0 | | | | |
| | 3d | 0 | 0 | 12 | | | | |

In order to discover a possible cause for the phenomenon just described, sections of the eye were made and examined microscopically. As expected, these sections showed that the pigment which imparts to the organ its color, is simply the pigment usually found surrounding the rhabdomes in the compound eye of arthropods. This pigment so far as its function is known, is supposed to be of a protective nature, placed so as to absorb all rays of light which do not fall directly parallel to the axis of the rhabdome in question. Of course, in cases such as we have under consideration the pigment is not black but colored, and will consequently reflect light of a certain wave length. At first,

TABLE 26
Vermilion eyed flies
 Group A

| SEX | | V | G | R | AVERAGE | | | |
|-----------|-----|---|----|----|-------------------------------------|--------|-------|------|
| | | | | | Sex | Violet | Green | Red |
| Male..... | 1st | 6 | 0 | 0 | Male..... | 90.4 | 72.0 | 59.6 |
| | 2d | 0 | 4 | 1 | Female... | 94.1 | 94.1 | 60.8 |
| | 3d | 0 | 2a | 5b | | | | |
| Female.. | 1st | 6 | 0 | 0 | Male { a. Tied once b. Tied once | | | |
| | 2d | 0 | 6 | 0 | | | | |
| | 3d | 0 | 0 | 6 | | | | |

Group B

| | | | | | | | | |
|-----------|-----|----|----|----|---|-------|------|------|
| Male..... | 1st | 3 | 0 | 0 | Male..... | 100.0 | 97.5 | 90.0 |
| | 2d | 3a | 3b | 1d | Female... | 95.0 | 72.0 | 76.6 |
| | 3d | 0 | 3c | 5e | | | | |
| Female.. | 1st | 6 | 0 | 0 | Male { a. Tied three b. Tied twice c. Tied twice d. Tied once e. Tied twice | | | |
| | 2d | 0 | 2 | 3 | | | | |
| | 3d | 0 | 4a | 3b | | | | |
| | | | | | Female { a. Tied once b. Tied once | | | |

Groups A and B combined

| | | | | | | | | |
|-----------|-----|----|----|-----|---|------|------|------|
| Males.... | 1st | 9 | 0 | 0 | Male..... | 95.2 | 84.7 | 74.8 |
| | 2d | 3a | 7b | 2d | Female... | 94.5 | 78.0 | 68.7 |
| | 3d | 0 | 5c | 10c | | | | |
| Female.. | 1st | 12 | 0 | 0 | Male { a. Tied three b. Tied twice c. Tied three d. Tied once e. Tied three | | | |
| | 2d | 0 | 8 | 3 | | | | |
| | 3d | 0 | 4a | 9b | | | | |
| | | | | | Female { a. Tied once b. Tied once | | | |

therefore, when normal eye color was thought to be the only one with an increased effectiveness for red, it seemed possible that this might be explained by assuming the red of the light to be exactly the same shade as that of the eye. This would then mean that a larger percentage of the red light entering the eye would be reflected and therefore effective, than would be the case with any other color. When it was discovered, however, that a still darker shade of red still further increased the effect-

TABLE 27
Normal eyed flies
 Group A

| SEX | | V | R | G | AVERAGE | | | |
|-----------|-----|----|----|----|-----------|----------|------|-------|
| | | | | | Sex | Violet | Red | Green |
| Male..... | 1st | 4 | 0 | 0 | Male..... | 100.0 | 96.2 | 97.0 |
| | 2d | 2a | 2b | 1d | Female... | 100.0 | 99.5 | 95.4 |
| | 3d | 0 | 4e | 5c | | | | |
| Female.. | 1st | 1 | 0 | 0 | Male { | Female { | | |
| | 2d | 3a | 4d | 0 | | | | |
| | 3d | 2b | 2e | 6c | | | | |
| | | | | | | | | |
| | | | | | | | | |

Group B

| | | | | | | | | |
|-----------|-----|----|----|----|-----------|----------|------|------|
| Male..... | 1st | 2 | 2 | 0 | Male..... | 93.7 | 93.3 | 86.6 |
| | 2d | 3a | 4d | 0 | Female... | 97.0 | 96.6 | 83.7 |
| | 3d | 1b | 0 | 6c | | | | |
| Female.. | 1st | 2 | 1 | 0 | Male { | Female { | | |
| | 2d | 4a | 5b | 0 | | | | |
| | 3d | 0 | 0 | 6 | | | | |
| | | | | | | | | |

Groups A and B combined

| | | | | | | | | |
|-----------|-----|----|----|-----|-----------|----------|------|------|
| Male..... | 1st | 6 | 2 | 0 | Male..... | 96.8 | 94.7 | 91.8 |
| | 2d | 5a | 6e | 1c | Female... | 98.5 | 98.0 | 89.5 |
| | 3d | 1b | 4f | 11d | | | | |
| Female.. | 1st | 3 | 1 | 0 | Male { | Female { | | |
| | 2d | 7a | 9d | 0 | | | | |
| | 3d | 2b | 2e | 12c | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |

iveness of that color this theory had to be given up. Thus, at the present time I have no explanation to offer for the increased effectiveness of red light which appears to accompany the darkening of pigment in the eye, other than the vague assumption that it may be due to some physiological difference which occurs in connection with this change of pigmentation.

TABLE 28
Sepia eyed flies
 Group A

| SEX | | V | R | G | AVERAGE | | | |
|-----------|-----|----|---|----|---------------------------------------|--------|------|-------|
| | | | | | Sex | Violet | Red | Green |
| Male..... | 1st | 5 | 1 | 0 | Male..... | 93.9 | 87.4 | 66.0 |
| | 2d | 1 | 5 | 0 | Female... | 93.9 | 91.0 | 68.0 |
| | 3d | 0 | 0 | 6 | | | | |
| Female.. | 1st | 3 | 3 | 0 | Female { a. Tied once b. Tied once | | | |
| | 2d | 2 | 3 | 0 | | | | |
| | 3d | 1a | 0 | 6b | | | | |

Group B

| | | | | | | | | |
|-----------|-----|---|---|---|-----------|------|------|------|
| Male..... | 1st | 6 | 0 | 0 | Male..... | 93.3 | 70.4 | 54.5 |
| | 2d | 0 | 6 | 0 | Female... | 96.6 | 86.6 | 55.0 |
| | 3d | 0 | 0 | 6 | | | | |
| Female.. | 1st | 6 | 0 | 0 | | | | |
| | 2d | 0 | 6 | 0 | | | | |
| | 3d | 0 | 0 | 6 | | | | |

Groups A and B combined

| | | | | | | | | |
|-----------|-----|----|----|-----|---------------------------------------|------|------|------|
| Male..... | 1st | 11 | 1 | 0 | Male..... | 93.6 | 78.9 | 60.2 |
| | 2d | 1 | 11 | 0 | Female... | 95.2 | 88.8 | 61.5 |
| | 3d | 0 | 0 | 12 | | | | |
| Female.. | 1st | 9 | 3 | 0 | Female { a. Tied once b. Tied once | | | |
| | 2d | 2 | 9 | 0 | | | | |
| | 3d | 1a | 0 | 12b | | | | |

SUMMARY AND CONCLUSIONS

1. Females of *Drosophila ampelophila* react to light somewhat more readily than do the males. This difference is most marked in young insects and steadily decreases with age, until at 8 or 9 days it has almost vanished. The time of maximum activity for both sexes does not seem to come at 18 hours, but more probably at from 3 to 5 days.

2. The removal of the wings causes the fly to lose most of its phototropism. The effect is specifically on the tendency to react to light, as is shown by the fact that such an operation

affects little if at all the response to gravity. The effect is roughly proportional to the amount of the wing cut off. It is not a result of the operation as such, since other operations do not produce it, and because wingless flies and flies of other stocks with defective wings show the same deficiency of response. Certain organs (fig. 3) occur on the wings of *Drosophila*, but operations fail to show that they are connected with the response

Showing relative tropism of flies of varied eye colors toward lights of different wave lengths



Graph 5

to light. It appears fairly certain on the other hand that the chief light receiving organs are the compound eyes as shown by experiments with eyeless stock.

3. Operations on the antennae may produce a weakening of the response to gravity, though they have little effect on the reaction to light.

4. In a mutant stock of flies known as tan, there is clear-cut evidence for the sex linked inheritance of a character which may be described as indifference to light. It is apparently not due to any structural defect in the eye.

5. Colored lights which may be conveniently described as violet, green and red, are effective in the order named upon insects whose eye color is lighter than the red eye of the wild fly. In the case of wild flies, and flies whose eyes are of a still darker shade called sepia, red is more effective than green.

GENERAL DISCUSSION

In most of the earlier work on various organisms, both animals and plants, the conclusion generally reached was that the blue and violet rays possessed much more stimulating value than did those which are less refrangible, particularly the red and orange. Thus Payer ('42) using both the solar spectrum and colored media found that seedlings turned toward blue and violet light but not toward red, yellow, orange or green. Sachs in 1864 obtained similar results, using colored solutions and glass. Also in the case of animals Engelmann ('82) found that *Euglena viridis* collected in the blue of a solar and gas spectrum, while E. B. Wilson ('91) working on *Hydra viridis* with colored glasses again found the maximum effect in blue. Finally, Loeb's earlier work ('90-'93) led him to conclude that as between red and blue, the latter color was the more effective for fly larvae, plant lice, caterpillars of *Porthesia chrysorrhoea*, moths of *Sphinx euphorbia*, *Geometra piniaria*, various copepods, the meal worm *Tenebrio molitor*, the larvae of *Polygordius*, *Limulus polyphemus*, and the June bug *Melolontha vulgaris*.

Even some of the early investigators, however, found cases in

which the above condition did not hold. Thus Kraus ('76) using colored media, discovered that in *Claviceps*, a fungus, red light was nearly as effective as blue, while Engelmann ('82) showed by the use of a solar and gas spectrum that *Bacterium photometricum* actually collects most readily in the infra red. Furthermore, the study of various other animals with more refined methods began to show that many forms were most affected by intermediate points in the spectrum. Thus Yerkes ('99) has shown that for *Simocephalus* the point of maximum efficiency in a Welsbach gas prismatic spectrum is in the yellow. Bert ('69) and Lubbock ('81) located this point for *Daphnia* in the green, while recently Loeb and Wasteneys ('16) using the spectrum from a carbon arc, have found the most effective point for *Balanus* larvae in the yellow and that for *Chlamydomonas pisiformis* in the yellow-green. Likewise, Hess, an ophthalmologist, ('10) using the spectrum from a Nernst glower has fixed green or yellow-green as the maximum stimulating point for a variety of forms, including ichneumon flies, *Culex pipiens*, adults and larvae, *Coccinella septempunctata*, *Dasychira fascelina* and cephalopods. In the last instance the reaction of the pupil of the eye is taken as a criterion of response. Lastly, S. O. Mast has recently given an excellent summary of work previously done and the results of a recent series of experiments of his own on *Arenicola* larvae, blowfly larvae and a number of unicellular forms. For the blow-fly larvae the maximum is in the green, while for *Arenicola* it is in the blue.

From these results it is apparent that the variation in the point of maximum response for different animals and plants is very wide. To explain this divergence, the existence in different organisms of different chemical compounds varying respectively in the degree to which they are altered by light of different wave lengths has been suggested. That there are compounds of this kind we know, but their presence in phototropic organisms has not yet been proved. Aside from this view Hess believes that phototropic animals are all color-blind, and that they go to the part of the spectrum which seems to them brightest. He apparently gets this idea from the fact that he found

so many organisms for which yellow-green is the most effective part of the spectrum, this being also the brightest part for color-blind men. This notion, has been criticized by Ewald ('15) and Loeb ('16).

The peculiar fact about *Drosophila* is the reversal in the effectiveness of red and green as the insects' eye color grows darker. Thus for eye colors lighter than normal the order of effectiveness is violet, green, red, while probably for normal, and certainly for sepia, the order is violet, red, green. This case besides showing the peculiar reversal is remarkable as being the only instance so far discovered among the lower animals in which red is more effective than green, with the possible exceptions of *Daphnia* (Frisch and Kupelwieser, '13; Ewald, '14), and paramoecium *bursaria* (Engelmann, '82). How to account for this phenomenon of reversal it is difficult to say. Were it not for the case of sepia it might be explained on the basis of the changed amount of red reflected by the normal colored rhabdomes as compared with that reflected by those of lighter shades. When two shades produce the same effect, however, it is difficult to see how this will suffice. It would thus seem as though we must fall back on the assumption that as the eye grows darker, the supposed sensitive chemical substances on which the light has its effect change also. What this change could be, it is hard to imagine from what we now know of photo-chemical reactions. I am inclined to think, therefore that the explanation may yet be found in connection with some sort of differential absorption.

It may be noted that my results with colored lights do not agree in one respect with those of Dr. A. O. Gross who also worked on *Drosophila*. This writer found green more effective than red for flies with normal eyes, while my experiments reversed this order. I suggest, however, that this discrepancy is due to the fact that Dr. Gross used lights which were equated in energy, whereas in the case of my filters, as is also true for the normal spectrum, the energy of the red is much greater than that of the green. This fact, nevertheless, does not invalidate or make less interesting the very evident increase in the effectiveness of red in the case of the darker eye colors, since whatever

the relative difference in energy content, that difference remained constant for all the eye colors tested in my experiments.

Lastly, it may be well to emphasize the peculiar relation which exists in *Drosophila* between general activity and phototropism. This phenomenon has been clearly recognized by Carpenter and in general I agree with this author's conclusions. The fact seems to be that this insect is not phototropic unless it is in a certain physiological state brought on by, or at least accompanied by, activity. When the fly reaches a certain degree of activity, induced by various means, it suddenly becomes phototropic. When it quiets down, however, it may still crawl about but ceases to be phototropic. Thus, when an insect has been exposed to constant illumination for some time, it no longer orients to light but wanders aimlessly up and down the tube. Eventually such an animal may even come to rest with its head away from the source of light. This phenomenon, Carpenter suggests, is probably due to slight fatigue. However this may be, it is certain that without a continuance of the mechanical agitation or sudden increases in light intensity, the animal's general activity soon falls to the point where phototropic response ceases.

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SOME EXPERIMENTS ON REGENERATION AFTER EXARTICULATION IN DIEMYCTYLUS VIRIDESCENS

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TEN FIGURES (THREE PLATES)

The earlier writers (Phillipeaux and Fraisse)¹ on regeneration in urodeles seem to have held the opinion that the extremities of adult animals are completely replaced only when one or more bones are injured in the amputation, that is to say, not after total extirpation (exarticulation). Wound-irritation from an injured bone was considered necessary as a stimulus to the replacement of a missing part. Also the bone supplied a 'tissue-rest' to serve as a matrix. However in young salamanders and especially in their larvae, it was found that the regeneration of extremities takes place very readily since here the joints are only partially formed and wounding of the bones always occurs in amputations. This general conclusion, that in adults regeneration does not occur after complete extirpation, seems to have been shared by a number of the more recent investigators, some, Kochs ('97) and Wendelstadt ('01 and '04) expressly confirming it, others, Towle ('01), Morgan ('03), Reed ('03) and Glaeser ('10), while not putting it to the test, seem to have taken care in their experiments to amputate through a bone.

Kurz ('12) in the course of his experiments on transplantation of entire limbs in Triton, found that if the limb is completely extirpated (exarticulated) at the hip- or shoulder-joint, a new limb regenerates. Presumably no wounding of the bones of the hip- or shoulder-girdle took place although Kurz does not state

¹ The works of Phillipeaux and Fraisse were not accessible to the writer. Their conclusions were obtained from Barfurth's review in Merkel and Bonnet's *Ergebnisse*, vol. 1, 1891.

what precautions were taken to avoid this. The writer using the American salamander, *Diemyctylus viridescens*, obtained similar results some years previous to Kurz's report but for various reasons they were not published.² Recently a new series of experiments were made to work out the histological details of the process and to determine how it differs, if at all, from regeneration after injury to remaining bones or cartilages. In addition, a number of more complicated operations were made to analyze further, if possible, the extent and power of regeneration after losses not usually met with in nature.

MATERIAL AND METHODS

A large supply of adult *Diemyctylus* was obtained through the kindly assistance of Prof. A. Treadwell, of Vassar College. Since many of these animals were in a weak, semi-starved condition when brought to the laboratory, they were kept for a month in glass aquaria before using and were fed on fresh liver. Under these conditions the animals became very vigorous, and withstood the operations well. All operations were done under narcosis. At first ether was used, but this, owing to its irritating effect on the skin and to a certain percentage of mortality which followed its use, was soon discarded. Much better results were obtained by using a solution of chloretone, of 1:2000, in which the animals were immersed. This acts very gently. After swimming around rapidly for a few minutes, the salamanders slowly come to rest and in about ten minutes are completely narcotized. The animals recover readily, though sometimes slowly after this treatment. There is no irritation of the skin and no mortality.

After the amputations, to be described in detail beyond, the best results were obtained by closing the wounds with a stitch or two of fine silk thread. Although this is not absolutely necessary to the success of the experiment, healing then takes place more rapidly and there is less danger of fungoid growths. Immediately after operation, the animals were placed in a dark

² The experiment was made at the suggestion of Prof. T. H. Morgan, in 1907.

chamber lined with moist filter paper for two days as recommended by Reed ('03). During this period the operated extremity was moistened from time to time with a solution of permanganate of potash 1:1000. The animals were then returned to the aquaria. The above precautions almost entirely prevented the growth of fungus and consequent failure of the experiments.

For microscopic study, the regenerating regions were removed and fixed for the most part in sublimate acetic or Gilson's mercurio-nitric fluid. Other fixatives, such as Zenker's fluid, Bouin's fluid and ten per cent formalin were occasionally used but on the whole the sublimate mixtures proved the most satisfactory. After hardening in alcohol for a few days, the objects were decalcified in a mixture of four per cent nitric acid in seventy per cent alcohol for three or four days. They were then imbedded in paraffin and sectioned. As a rule, good series were obtained, seven or eight micra thick, although the rather tough bone and cartilage from large specimens sometimes gave trouble. For staining Mayer's haemalum followed by picro-acid fuchsin was most frequently employed. This gives a brilliant differentiation of tissues but is not always permanent. Other stains such as Mallory's connective tissue stain, borax carmine and Lyons blue, haemalum and congo red were also used but none proved as satisfactory for most purposes as the haemalum and picro-acid fuchsin combination.

EXPERIMENTS

Pari I

The fact that regeneration does occur after complete extirpation (exarticulation) has been established by the observations of Kurz and the writer as stated above. In order to work out the detail of this process, two sets of operations were made, the hind limbs being used in both cases. In the first set the limb was amputated at the hip-joint, in the second at the knee-joint. Great care was exercised in making these amputations. The skin and muscles were first carefully divided with a small sharp

scalpel. Then the part to be removed was grasped with the forceps and slight traction employed to draw the joint surfaces apart. The capsular ligaments were then divided with the scalpel and the limb removed, care being taken not to touch the skeletal parts remaining (hip bones or femur according to the site of operation). A flap of skin and muscle was drawn over the wound and a couple of stitches taken. There was very little trouble from bleeding, but in cases where it was profuse, the specimen was discarded.

1. *Amputation at the hip-joint.* Nineteen animals were used for this operation divided into groups of eleven, six and two. All of the first group were killed and examined between thirty-nine and forty-six days after operation. Externally each showed a small bud at the site of amputation. Microscopically the bud was composed of a dense mass of indifferent cells with small round nuclei. No change in the hip-girdle was observed. The second group of six were kept for six months. At this time all had regenerated a new limb about three-fourths the normal size. Microscopic examination showed the normal number of skeletal elements in the limb, each represented by a bar of cartilage. There was a well developed narrow cavity in the femur and peripheral ossification had begun in all the cartilages except the tarsals which do not ossify in these animals.³ Joint cavities were well marked at this time. The third group of two animals was lost. Owing to the small number of specimens and the lack of intermediate stages, the successive steps in this type regeneration could not be made out. The detailed account of this process will therefore be based upon the larger and more complete series of operations at the knee-joint (vid. infra).

2. *Amputation at the knee-joint.* About seventy-five operations of this kind were made. Most of the specimens obtained were fixed at intervals, of from ten to fifty days and sectioned. The remainder were allowed to complete their regeneration to

³ While it is true that peripheral ossification does not occur in the tarsalia, nevertheless an extensive marrow cavity is normally present and the irregular trabeculae of cartilage bordering it generally become calcified, if not actually bony.

determine whether the new part exactly resembles the old both in size and gross structure. In addition fifteen amputations were made through the distal end of the femur for comparison with the exarticulation experiments.

Descriptive. Wendelstadt ('04) and Glaeser ('10) have given very detailed accounts of regeneration in the limbs based chiefly on species of the European salamander *Triton* and on the *Axolotl*. In *Diemyctylus* the process is quite similar to that observed in *Triton*. A study of the specimens in which amputation was made through the distal end of the femur, i.e., an operation corresponding to those of Wendelstadt and Glaeser, showed that the descriptions given by these writers for *Triton*, apply almost equally as well to *Diemyctylus*. It is true there is some slight discrepancy in their accounts but this can be discussed more conveniently when comparing the exarticulation experiments with those previously made.

The earlier changes which take place in the stump may be passed over briefly here. They are concerned chiefly with the over-growth of the integument, the breaking down of the soft parts, notably the muscle and the formation of a dense mass or bud of small cells with round, deeply staining nuclei over the distal end of the bone (femur). This mass lengthens out and forms a projection when seen externally but does not always lie in the axis of the limb. The origin of these cells could not be determined with exactness. Wendelstadt ('04) encountered the same difficulty. Towle ('01) however, states that the accumulation of 'nuclei' in the bud is due to rapid (direct) division of nuclei in the old muscle fibers and the disintegration of these fibers.⁴ Undoubtedly the degeneration of muscle-fibers is largely responsible for the accumulation, but whether exclusively so or not, is difficult to decide. Connective tissue elements may also contribute something.

Turning now to the changes in the bone and cartilage with which this paper is chiefly concerned, it is here that an essential difference appears between regeneration after exarticulation and

⁴ Towle's experiments are concerned almost entirely with the regeneration of muscle.

regeneration after amputation through a bone. This difference has to do principally with the behavior of the distal epiphyseal cartilage of the femur. This epiphysis (fig. 1, *Ep.f.*) becomes slowly detached from the shaft by resorption of the bone and calcified cartilage (*C.c.*) proximal to it.⁵ The resorption seems to be brought about largely by the action of cells which arrange themselves along the surface of the bone or calcified cartilage and erode it. Many of them are giant cells. Their origin was not determined. Both Wendelstadt ('04) and Glaeser ('10) have described this resorption process by giant cells. Figure 1 shows the distal end of the shaft (*B.f.*) broken up into irregular fragments of bone and calcified cartilage.

Coincident with the resorption process, a change takes place in the epiphysis itself. The cartilage matrix begins to break down. This occurs first on its distal and proximal surfaces (fig. 1, *Ep.f.*). There is no evidence, however, that the cartilage cells themselves undergo degeneration. Indeed, in many instances they have been seen dividing mitotically and further, as the lacunae are opened by the degeneration of the matrix, the cells pass out and mingle with the surrounding tissues. Those liberated on the distal surface could not be further traced but on the proximal surface, that is facing the marrow cavity (figs. 1 and 2, *M.C.*), they contribute to a mass of tissue (*Ax.C.*) which is forming between the old epiphysis and the shaft. This mass which quickly takes on the appearance of young cartilage may be called axial cartilage adopting Glaeser's ('10) term.

This axial cartilage appears to have a two-fold origin: (a) from the liberated cells of the old epiphysis as stated above and (b) from the cells lining the marrow cavity and covering the trabeculae of bone and calcified cartilage at the distal end of the shaft; in other words from the endosteum. These latter cells appear to increase in number and, streaming out from the interior of the femur (fig. 1) become massed under the old epiphysis where they form the main contribution to the new axial cartilage.

⁵ It may be stated that there is normally a considerable amount of calcified cartilage at the plane of union of shaft and epiphysis. This is distinct from the uncalcified, hyaline cartilage of the epiphysis itself.

This substantiates Wendelstadt's conclusion that 'osteoblasts' (in reality chondroblasts) arising from the lining of the marrow cavity contribute to the new formation (of cartilage). Glaeser, however, derives the axial cartilage from periosteal and connective tissue fibrillae exclusively. He thinks that cartilage may occasionally arise from bone-marrow though this is doubtful. The origin of a part of this cartilage from cells of the old (distal epiphyseal) cartilage naturally could not occur in the experiments of Wendelstadt and Glaeser since the distal epiphysis was always removed in their operations. Glaeser nevertheless states that regeneration from old cartilage, either from the cells or the matrix does not occur. The writer is unable to determine from Glaeser's account upon what experiment this conclusion is based. That giant-cells in certain regions break down the matrix and open up the capsules is true but in *Diemyctylus*, at least, the cartilage cells certainly do not degenerate. Evidences of further activity are abundant in this material including the frequent presence of mitosis.

During the early stages in the formation of axial cartilage, the cells of the periosteum also begin to form cartilage. The site of this new formation is at first some distance from the distal end of the femur and entirely distinct from that of the axial cartilage. It may be called periosteal cartilage, or using Glaeser's term, peripheral cartilage. An early stage of its growth is shown in figure 1, *Per.C*; a later one in figure 2, *Per.C*. The formation of peripheral (periosteal) cartilage is mentioned by most workers on regeneration and there is no need to discuss it at length. In all cases it first appears at some distance proximal to the wound and gradually spreads distalward until it forms a continuous collar around the shaft reaching to the distal end (fig. 3, *Per.C*). Cornil and Coudray ('03) described a similar formation in the healing of experimentally produced fractures in a mammal (rabbit). The first phenomena of repair, they state, are to be found at some distance from the fracture, that is the formation of peripheral (periosteal) cartilage.

Whether or not the axial cartilage always appears first could not be determined. In some cases the peripheral cartilage was

distinguishable earlier while in most the two appeared at about the same time. Glaeser seems to think the axial cartilage appears before the peripheral, while Wendelstadt describes activity in the periosteum leading to the formation of peripheral cartilage before there is any outgrowth of cells from the marrow cavity to form axial cartilage. In the experiments on *Diemyctylus* the presence of an epiphysis may have had a modifying effect on the order of appearance. This point is probably not of any great importance.

Soon after the appearance of axial and peripheral cartilage the tissue of the bud which has formed distal to the femur, begins to show signs of differentiation. This tissue as mentioned previously is at first composed of apparently indifferent cells. Cartilaginous masses now appear in it and, since they are formed in the same manner as in normal development, they may be spoken of collectively as embryonal cartilage following Glaeser ('10). In figure 2, a mass of this cartilage (*Em.C.*) can be seen lying distal to the old epiphysis (*Ep.f.*). It is at first entirely distinct from the axial cartilage.⁶ As the limb bud enlarges rapidly the growth of the embryonal cartilage keeps pace with it, the cartilage extending right into the growing tip (fig. 3, *Em.C.*) In this way the skeleton of the new leg and foot is blocked out in cartilage at a young stage, as the early experimenters found.

The formation of new skeletal parts is due chiefly to concentrations of cells in situ rather than to growth from the first formed mass or masses. There is, it is true, a continuous substratum or core of tissue running through the center of the bud from which the skeleton arises but it does not give rise to one or two continuous bars of cartilage. There are always interruptions at points where joints are to be formed.

During the growth of the embryonal cartilage, the axial cartilage continues to enlarge. This is due in part to growth at the expense of the old epiphyseal cartilage. The latter remains, however, for some time partly imbedded in the axial cartilage (fig. 3, *Ep.f.*) where it can be distinguished by its different

⁶ The space seen in figure 2, between the embryonal cartilage and the old epiphysis is probably an artifact.

staining reaction. The expansion of the axial cartilage finally brings it into contact with the embryonal cartilage and they become so closely united as to appear almost continuous (fig. 3).

The peripheral cartilage (fig. 3, *Per.C.*) meanwhile has spread to the distal end of the bone (*B.f.*) and there unites with the axial cartilage (*Ax.C.*). This results in a continuous cap of cartilage covering the distal end of the femur, and extending proximally for some distance. Figure 4, *Ep.f.n.* from a much later stage than figure 3, shows this new cap. From it, the new epiphysis of the femur is formed and also the new bone of the intermediate zone between epiphysis and shaft which was lost in the early resorption process. A portion of the marrow cavity *M.c.n.* can be seen extending into it.⁷ At *Os.n.*, new bony tissue is spreading into the cartilage. This new ossification is shown under high magnification in figure 10, which is taken from the same region of a neighboring section. The new bone (*Os.n.*) appears as a mass of interlacing fibers extending through the matrix of the cartilage (*C.n.*). In this process, the cartilage cells are gradually enclosed by the newly formed bone and converted directly into bone cells. Three such cells are shown at *Os.c.* Portions of the marrow cavity (*M.C.*) and bone of the shaft (*B.f.*) are included in the figure. Between the shaft and the marrow cavity some of the old calcified cartilage remains (compare fig. 3, *C.c.*). A direct transformation of cartilage into bone was noted by Cornil and Coudray ('03) in the healing of fractures in the rabbit. They state that in most cases the cartilage bordering the bone first ossifies along its edges while later the cartilage capsules themselves ossify, the cells being directly transformed into bone cells.

It will be recalled that a portion of the distal end of the shaft is destroyed in the resorption process by which the old epiphysis is detached from the bone. This is replaced by ossification of a part of the new cartilage in the manner just described. The further history of this process was not followed owing to lack of material in the advanced stages.

⁷ Through an error, the leader from the letters *M.c.n.* does not point to this extension.

Distally the new formed epiphysis comes into relation with the embryonal cartilage which forms the new fibula and tibia (figs. 4 and 9) and later a joint appears at this level. As previously stated, before the new epiphysis is completed and while a portion of the old epiphysis is still present (fig. 3) a close attachment is developed between the axial cartilage (*Ax.C.*) and the embryonal cartilage (*Em.C.*). It is difficult to say whether or not the two cartilages become actually continuous. The tissue uniting them is composed of cells without definite boundaries and with small elongated nuclei. It does not appear to be cartilaginous. Small cavities soon appear in this connecting tissue, beginning usually at the circumference and spreading toward the interior. An early stage in the joint formation is shown in figure 9. The new joint cavity (*J.c.n.*) is just appearing between the new femoral epiphysis (*Ep.f.n.*) and the new fibula (*Fib.n.*). In a later stage (fig. 4) the joint cavity has enlarged at the circumference and a definite capsule has been formed, the latter being continuous with the perichondrium of the new cartilages (epiphyses). A small joint cavity has also developed between the fibula and a tarsal cartilage (*T.c.n.*). At this stage the fibula itself has begun to show evidences of subdivision into shaft and two epiphysis. In the central portion, the cartilage areolae are enlarging and becoming more spherical preparatory to the formation of a marrow cavity, while at the surface a thin layer of subperiosteal bone has been laid down. In the epiphyseal portions, the cartilage cells have a tendency to arrange themselves in concentric arcs, a characteristic of epiphyses in general.

No attempt has been made in this study to arrange the specimens in a series based on time after operation. The stages of regeneration do not necessarily correspond to the intervals of time. For example figure 3 shows a stage obviously more advanced than that shown in figure 2, yet it was taken from a specimen killed thirty-eight days after operation, while the latter came from one killed at forty days. This is very probably due partly to difference in time of healing of the wound and partly to temperature. It was noticed that among animals operated

on at the same time and as far as possible treated in the same manner, some always healed more readily than others. Regarding temperature—the higher degrees were in general more favorable to rapid regeneration than the lower as might be expected, but there was so much individual variation due to time of healing that no great reliance can be placed on this statement. Comparing, from the standpoint of time, regeneration after exarticulation with regeneration after wounding a bone, one finds that the former is appreciably slower. On the average, there is about ten days difference in corresponding stages.

With regard to regeneration of soft parts (muscle, nerve, blood-vessels, etcetera), there is nothing to add to the accounts already published. Muscle regeneration has been very carefully worked out by Towle ('01) in *Plethodon* and Schminke ('07) in *Triton taeniatum* and *T. cristatus*. These writers agree that the new muscle is formed chiefly by isolated cells (sarcoplasts) which arise from degeneration of the old muscle of the stump. The sarcoplasts are small masses of cytoplasm which contain at first several nuclei. They usually break up into small cells which are responsible according to Towle ('01) for the accumulation of nuclei (cells) in the growing bud distal to the stump of the old bone where they give rise to new muscle fibers. It was previously mentioned that the exact origin of this entire mass of small cells is somewhat uncertain (cf. Wendelstadt). Probably most of the cells originate from degenerating muscle and some perhaps from other soft tissues. In any event, there is no evidence that any are derived from bone or cartilage. Histologically there is at first no sign of differentiation in these cells, and it seems useless to assume that such exists. It is from a part of them, however, that new embryonal cartilage is developed in the midst of the bud. This seems to be an example of dedifferentiation followed by redifferentiation in the sense of Child ('15). From the apparently indifferent mass both cartilage and muscle are formed, the cartilage showing the typical embryonic type of development.

A somewhat similar process is to be seen in the behavior of the old epiphyseal cartilage of the femur. Here the matrix breaks

down, liberating the cells which again become active and form new matrix. In this case, however, the cells do not dedifferentiate so far as to become indifferent; they remain cartilage forming cells. The formation of the peripheral cartilage and that part of the axial cartilage derived from endosteal cells is of course quite different in nature. Here the more or less undifferentiated cells of the periosteum and endosteum which have lain dormant, are suddenly stimulated to activity by the amputation. They form cartilage first and later a portion of the cartilage is transformed into bone as described on page 115.

It was stated at the beginning of this account that some animals were allowed to complete their regeneration to determine whether the new skeleton was like the old. There seems to be no difference whatever provided sufficient time is allowed for development. Externally, a slight deformity sometimes appears at first, since the new bud does not always lie in the longitudinal axis of the limb. This is more common after operations at the knee-joint than at the hip-joint. In the course of time this irregularity disappears and the limb becomes normal in shape and position. Complete ossification, however, may take almost a year and sometimes even longer.

A glance over the literature of regeneration in amphibia shows that the power to regenerate a new normal skeleton does not extend to all animals of this class. Morgan ('03) found that in *Amphiuma* the new skeleton was abnormal and deficient although some specimens were kept under observation for nearly a year. Certain results which were obtained by the earlier experimenters Goette and Fraisse seem to indicate that some of the European urodeles (*Triton marmoratus* and *Proteus*) lack the power of complete regeneration but Kammerer ('06) states that this is not the case if the animals are kept under favorable conditions and for a sufficient length of time.

In the *Anura* the power is much more limited. New limbs will regenerate only if amputation is made in the tadpole stage. Barfurth ('94) was the first to find that the limbs of frog-larvae (*Rana fusca*) are capable of regeneration, but this power disappears in the progress of development. Ridewood ('98) obtained

regeneration of posterior limbs in the tadpole of the midwife-toad (*Alytes obstetricans*). The new skeleton was "normal or nearly so" in five cases. Byrnes ('04) using frog-tadpoles showed that the anterior limbs would regenerate while still under the operculum but the new limb is invariably smaller than normal and there is a tendency to reduction in the skeletal elements. Morgan ('08) (and Goldfarb) attempted to induce regeneration in the fore-leg of the adult frog by artificial means. Pieces of the leg, muscle and other tissues from the tail of the tadpole were grafted into the stump but with only small success. In some cases, however, incomplete regeneration of the leg with rudimentary toes was obtained, or a broad flat 'foot' with scant toes. Histological details were not given. Glaeser ('10) more recently tested the power of regeneration in the hind limbs of adult frogs but found none except in two cases where a ring of peripheral (periosteal) cartilage developed around the stump of the femur. No artificial means were used to induce regeneration in this case.

Part II

To test further the power of regeneration in *Diemyetylus* a series of more complicated operations were made, involving losses not usually met with under natural conditions. These are to some extent a repetition of those of Wendelstadt ('01) and Reed ('03) but with certain modifications.

Experiment 1. Extirpation of the fibula and a portion of the femoral epiphysis but without injury to the tarsus. Number of animals, ten:—Of these two escaped and one lost the foot. The remaining seven were killed at intervals of from forty-eight days to one year. There was no indication of regeneration of a new fibula, but the lost portion of the femoral epiphysis was restored.

Experiment 2. Extirpation of the fibula without injury to either femoral epiphysis or tarsus. Number of animals, ten:—These were killed at intervals of from thirty-three days to one year. For the most part no indication of regeneration was observed but in two specimens there was a narrow mass of cal-

cified fibrous tissue or bone in the place occupied by the old fibula. This mass may have developed from fragments of the old fibular epiphysis which were accidentally left in the wound in two of the operations.⁸

The results of experiments 1 and 2 substantiate the conclusion of Wendelstadt and Reed that regeneration in a lateral direction (in the limb) does not occur.

Experiment 3. Extirpation of both leg bones and a portion of the femoral epiphysis but without injury to the tarsus. Number of animals, five:—Considerable shortening occurred but in no case did the foot drop off. One was killed after sixty-three days and the remainder after one year. Two of the latter showed some indication of regeneration. In these there were one or two cartilaginous nodules, in one case fairly extensive, connected with the femoral epiphysis by fibrous tissue. The femoral epiphysis itself was restored in all.

Experiment 4. Extirpation of both leg bones but without injury to either femoral epiphysis or tarsus. Number of animals, seven:—Shortening of course occurred. Two lost the foot subsequently and were discarded. Of the five remaining, one was killed at sixty days and the remainder after one year. All showed definite attempts at regeneration, in some cases quite well marked. Figures 5 and 6 from the same specimen cut in the dorsi-ventral plane show the extent of regeneration in the best marked case. The time elapsed was sixty days. Only a small portion of the femoral epiphysis (*Ep.f.*) and one tarsal cartilage (*T.c.*) appear in figure 5, since the section lies near the border of the limb. The new skeletal element of the leg (*L.s.n.*) consists of an epiphyseal portion which articulates with the femur and a long, narrow bar of cartilage which is partly overlaid by bone at either end. (The bone is darkly shaded in the figure.) At the proximal end a distinct joint capsule with cavity (*J.c.n.*) has developed. Figure 6, from a section near the median plane, shows another portion of the new skeletal element (*L.s.n.*). This portion is

⁸ In the experiments described in part II, all bones removed were examined under a binocular microscope to determine whether any part of them had been accidentally left in the wound.

a solid mass of cartilage united to the femoral epiphysis (*Ep.f.*) by a capsule with joint cavity (*J.c.n.*) as before. Distally it is in contact with a tarsal cartilage (*T.c.*) but no joint capsule has developed. The two portions of the new element are continuous proximally when traced through the series. Unfortunately intermediate stages in the formation of the new skeletal element were not obtained. The epiphysis of the femur (fig. 6, *Ep.f.*) has the appearance of new cartilage similar to that of the new element (*L.s.n.*). This seems to indicate that the regeneration was centrifugal in direction and probably occurred in the same manner as described in the first section of this paper. No changes were observed in the tarsal cartilages.

Figure 7 is from another specimen killed after one year and cut in a plane passing through the borders of the limb. The new elements here consist of two large masses of cartilage (*L.s.n.*) united by fibrous tissue and connected with femoral epiphysis (*Ep.f.*) by a capsule containing a joint cavity (*J.c.n.*). Distally the new cartilages fall short of the tarsus. The tarsal cartilages themselves (*T.c.*) show signs of growth in a proximal direction (centripetal regeneration). They have become united proximally by a mass of cartilage which, however, has no connection with the new skeletal elements. The arrangement produces what may be called a soft joint.

Other specimens in this experiment showed the formation of irregular masses or nodules of cartilage but not so extensively as the two described above. There appears, then, to be a limit to the power of regeneration under the conditions of the experiment. This may be due to an inhibiting influence from the presence of the foot and to shortening of the limb which leaves very little room for the new growth. It may be well to state that as soon as the wound heals, the animal uses the limb constantly when creeping over the bottom of the aquarium. Wendelstadt ('01) performed a similar experiment upon the anterior limb of the Axolotl but with entirely negative results though he kept the animals under observation for ten to fifteen months. The limbs shortened as in the case of *Diemyctylus* but the animals apparently made no attempt to use them. It is just possible

that a certain amount of activity in the limb is necessary to start the regenerative process. Wendelstadt also tried the effect of leaving a small piece of one of the bones (ulna) in situ. For this operation he used one Axolotl and one Triton. In the latter the humerus was also wounded. The axolotl regenerated a new ulna which was shorter than normal while in the Triton a whole new forearm and a second hand were formed. This peculiar malformation in the Triton was never duplicated in any of the writer's experiments on *Diemyctylus*, although in some cases the femur was purposely wounded. It is improbable that there is any difference in the power of regeneration of fore and hind-limbs in these animals.

Experiment 5. Extirpation of the fibula and removal of the foot entire without injury to the femoral epiphysis or tibia. Number of animals, five:—In this lot two were killed at sixty-six and ninety-five days respectively and the remainder at the end of a year. The first of these had regenerated a well-marked foot when killed and a new fibula. The latter consisted of a solid bar of cartilage with a layer of subperiosteal bone surrounding its proximal two-thirds. It was attached to the femoral epiphysis by a capsule, common to it and the tibia. Distally it was connected with the new tarsalia by ligaments, in places showing the beginning of a joint cavity. In the specimen killed at ninety-five days, the foot had regenerated but there was scarcely any indication of a new fibula. The remaining three specimens killed after one year regenerated a new complete foot including tarsalia and a new but incomplete fibula. A section through one of these is shown in figure 8. The old tibia (*Tib.*) articulates with the femoral epiphysis (*Ep.f.*) while the new fibula (*Fib.n.*) falls short proximally. Peripheral ossification has started in the new element but there is no marrow cavity as yet. The new tarsalia are seen at *T.c.n.* The distal epiphysis of the tibia seems to be composed of new cartilage like that of the tarsals and fibula. This is to be expected since it was shown that the old epiphysis in a stump is always replaced by new cartilage (vid. Part I). In the present experiment one would expect first a new formation of cartilage from which a new tibial epi-

physis and the skeleton of the new foot is formed. This is followed by growth in a proximal direction to form the new fibula (centripetal regeneration). Apparently the energy of regeneration is not always sufficient to produce a complete fibula even in a year's time although it may do so in two months as in the first specimen described (sixty-six days).

A tendency to regenerate centripetally was also noted in experiment four, where both leg bones were removed and the foot allowed to remain. In this case however it was limited to the formation of a mass of cartilage (fig. 7, *T.c.*) uniting the proximal surfaces of the tarsalia. Wendelstadt ('01) obtained centripetal regeneration in the axolotl by extirpating the upper ends of the radius and ulna. In three animals, the bones were completely restored after fifteen to eighteen months. There was apparently no tendency to regenerate centrifugally from the femoral epiphysis.

Morgan ('08) using *Plethodon* and *Diemyctylus* tried to discover what kind of a structure would regenerate from the proximal end of a limb. For this purpose the limb was cut off and grafted onto the stump in reverse position. Regeneration occurred but results were complicated, due to mixing of old and new material and to the turning of the graft in the skin-pocket. This method was discarded in favor of the following: The hind leg was cut off at the knee. Then the femur was cut off high up in the thigh and the distal portion reversed in position. A new limb regenerated. Its skeleton was composed of (1) proximal stump of femur, (2) connecting cartilage, (3) piece of reversed femur, (4) new tibia and fibula, and (5) foot. There was considerable evidence of absorption and in only a few cases did it seem probable that the material for the new limb came from the exposed proximal end of the grafted piece. In some cases the new cartilage from the proximal stump grew past the graft. On the whole it is not quite clear from Morgan's account what part the graft played in regeneration, as histological details are not given.

Reed ('03) performed a series of experiments somewhat similar to those just described (Exp. 5) except that the distal end of the

tibia was removed with the foot. *Spelerpes ruber* was used for these experiments. The results were, regeneration of the distal end of the tibia, a new foot and a new fibular element. The latter was usually incomplete but in one case it almost completed itself proximally, that is, reaching the femoral epiphysis. As in the present experiments there was no tendency to regenerate from the femur. Wendelstadt, in his later paper ('04) states that the experiments of Reed confirm his general conclusion that wounding of the skeletal elements is necessary for regeneration. The experiments described in the present paper show that this conclusion is too sweeping. It is true that if one bone only (fibula or radius) is removed (Wendelstadt, Reed and the writer) or if the proximal parts of two bones are removed (Wendelstadt) no regeneration occurs from the uninjured epiphysis of the femur (or humerus). In the first case, the pressure of the remaining bone against the joint surface of the femur (or humerus) and the tarsals (or carpals), that is the presence of a functional joint may inhibit regeneration from these points. In the second case the new growth centripetally from the remaining injured bones, which is always more rapid than from uninjured ones, may make up the deficiency in time to check any tendency to regenerate from the epiphysis of the humerus. Shortening of the limb which must occur in this case would also be a factor. These, of course, are mainly suggestions. Further experiments are necessary before definite explanations can be made.

SUMMARY AND CONCLUSIONS

1. In *Diemyctylus* regeneration takes place readily after complete extirpation (exarticulation) whether the operation is made at the hip- or knee-joint (Part I), or at the ankle-joint (Part II, Exp. 5). The time elapsed is somewhat longer than when a skeletal element is injured.
2. The new skeletal elements are similar to the old. There is no tendency to reduction.
3. The essential difference between regeneration after exarticulation and regeneration after wounding a skeletal element lies in the behavior of the cartilaginous epiphysis which is present

in the stump in the former case. This cartilage becomes detached from the shaft, gradually breaks down and is, partly at least, reconverted into cartilage which assists in the formation of a new epiphysis.

4. The new cartilage which forms the basis for the skeletal elements appears independently in three localities:

a) Around the shaft of the bone proximal to the epiphysis (peripheral cartilage). This cartilage is periosteal in origin.

b) In the axis of the bone and in contact with the marrow subsequent to detachment of the epiphysis (axial cartilage). The origin in this case is twofold: (1) From the cells of the old epiphyseal cartilage and (2) from the lining of the marrow cavity (endosteum).

c) In the tissue of the bud distal to the epiphysis (embryonal cartilage). Here dedifferentiation appears to have taken place forming a substratum of indifferent cells from which in turn new cartilage is formed as in early development of the limb.

5. If a single bone (fibula) is removed completely from the leg, it is not replaced either by proliferation from its fellow (lateral regeneration) or from the skeletal elements lying proximal and distal to it even when one of the latter is injured.

6. When both leg bones are completely removed they are replaced to some extent by new elements which, however, are always irregular and incomplete. The origin of the new parts was not definitely determined.

7. When one leg bone (fibula) and the foot are removed without injuring any of the remaining skeletal elements, a new complete foot is regenerated from the distal end of the remaining leg bone (tibia). This is followed by a slow and often incomplete regeneration of the lost leg bone (fibula) in a proximal direction (centripetal regeneration).

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PLATES

PLATE 1

EXPLANATION OF FIGURES

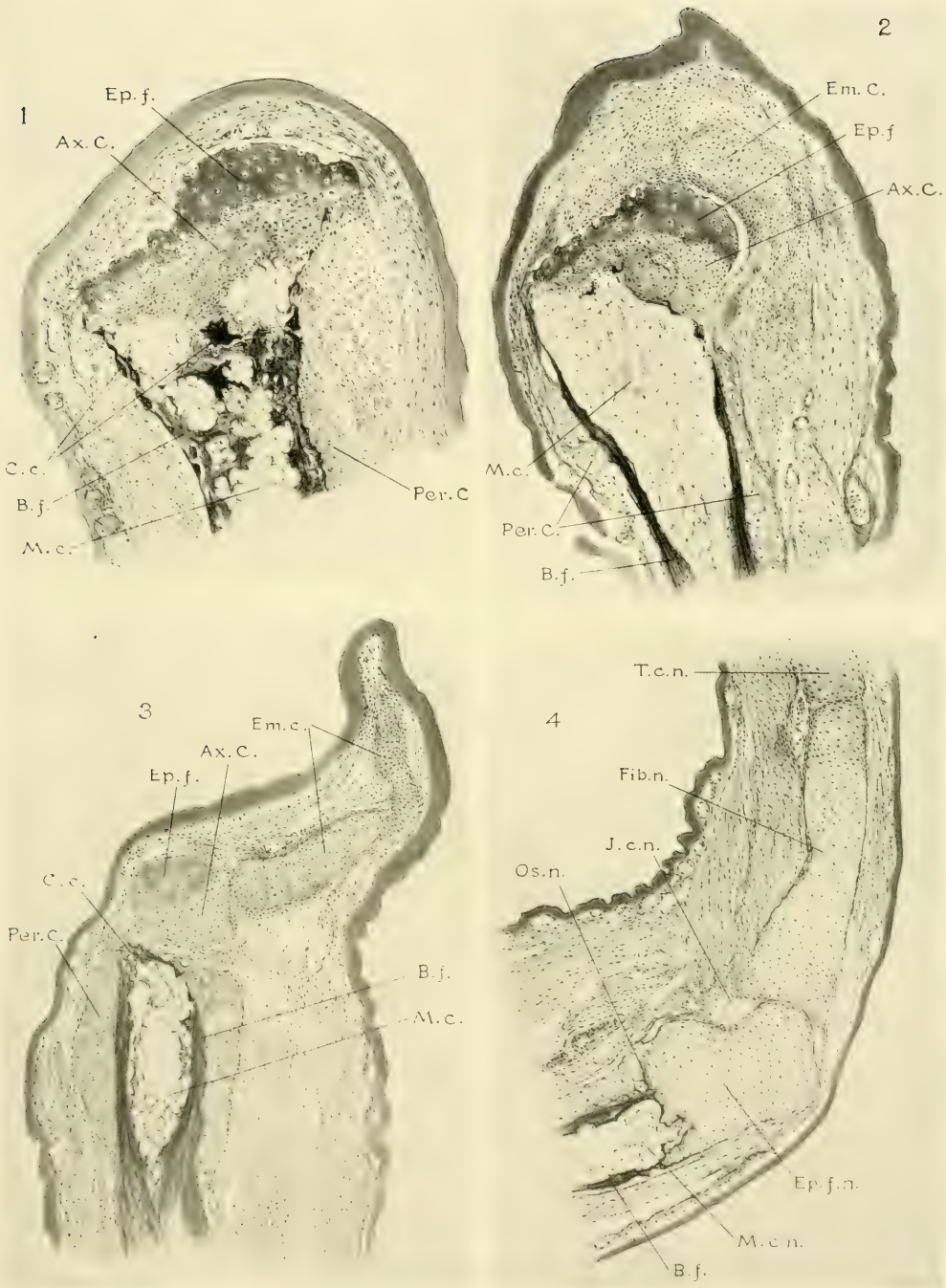
Figures 1 to 4 are from specimens in which complete amputation of the limb was made at the knee-joint.

1 Longitudinal section of a limb 30 days after operation. *Ep.f.*, femoral epiphysis; *Ax.C.*, axial cartilage; *Per.C.*, peripheral cartilage; *C.c.*, calcified cartilage of the shaft; *B.f.*, bone of the shaft (femur); *M.c.*, marrow cavity. The narrow space distal to the epiphysis is an artifact. Magnified about 30 diameters.

2 Longitudinal section of a limb 40 days after operation. *Em.C.*, embryonal cartilage. Other abbreviations as in figure 1. The space between the femoral epiphysis, *Ep.f.*, and the embryonal cartilage *Em.C.*, is probably an artifact. Magnified about 30 diameters.

3 Longitudinal section of a limb 38 days after operation. Abbreviations as in figures 1 and 2. Magnified about 30 diameters.

4 Longitudinal section of a limb 48 days after operation. *T.c.n.*, new tarsal cartilage; *Fib.n.*, new fibula; *J.c.n.*, new joint-cavity; *Ep.f.n.*, new femoral epiphysis; *Os.n.*, bone formation in new cartilage; *M.c.n.*, extension of the marrow cavity into the new cap of cartilage (see foot-note on p. 115); *B.f.*, bone of the femur. Magnified about 30 diameters.



H. Murayama del.

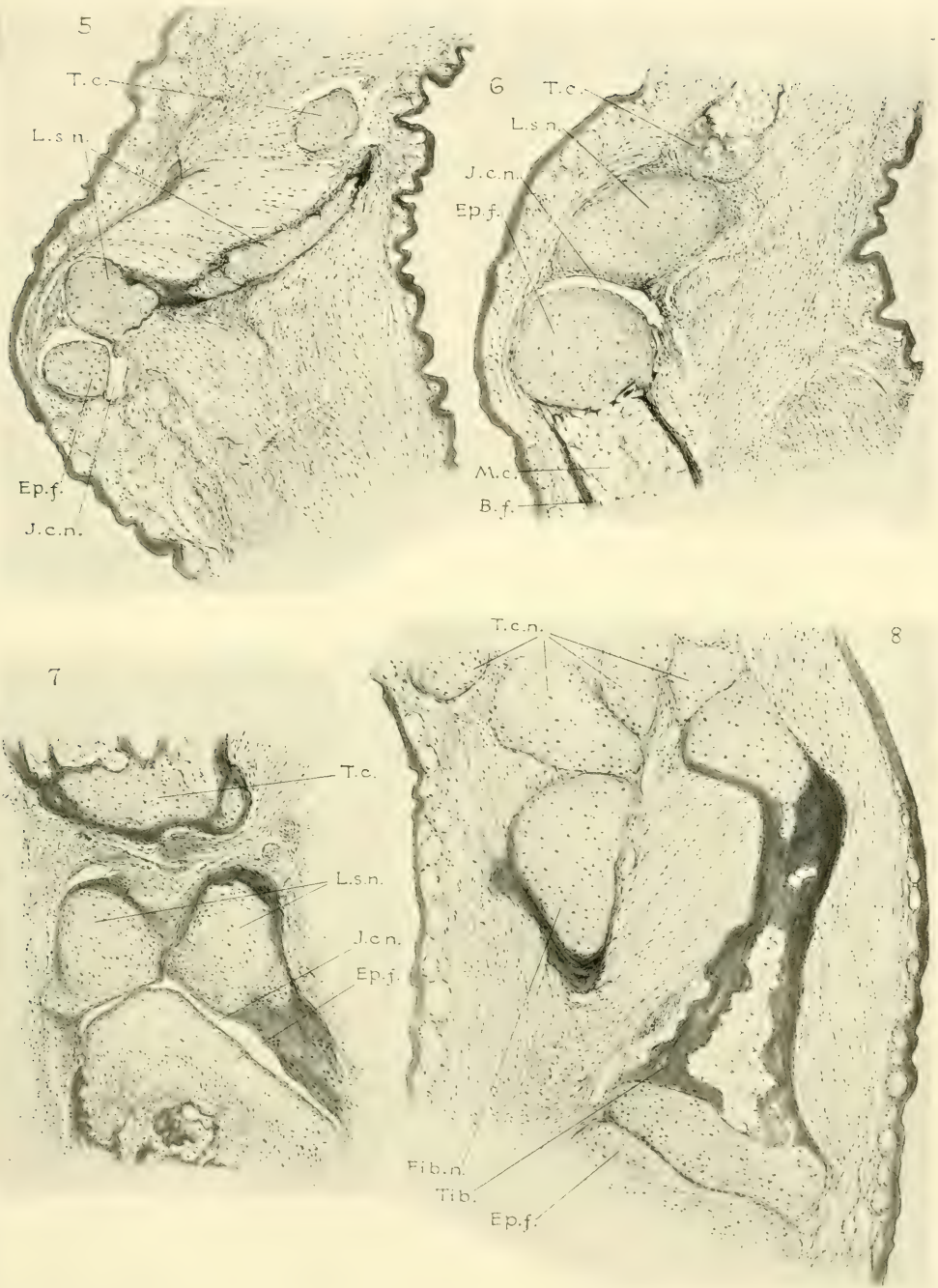
PLATE 2

EXPLANATION OF FIGURES

5 and 6 Longitudinal sections of a limb from which both tibia and fibula were completely removed. Time after operation, 60 days. *T.c.*, tarsal cartilage showing normal marrow cavity and partly calcified lining; *L.s.n.*, new skeletal element of the leg, in figure 5, partly ossified; *Ep.f.*, epiphysis of the femur; *J.c.n.*, new joint cavity; *M.c.*, marrow cavity and *B.f.*, bone of the femur. Magnified about 30 diameters.

7 Longitudinal section of another specimen after same operation as above. Time one year. Tarsal cartilages blended proximally, *T.c.*; new skeletal elements *L.s.n.* Other abbreviations as in figures 5 and 6. Magnified about 30 diameters.

8 Longitudinal section of a limb from which the foot and the fibula were completely removed. Time after operation, one year. *T.c.n.*, new tarsal cartilages; *Fib.n.*, new fibula (incomplete); *Tib.*, tibia; *Ep.f.*, femoral epiphysis. Magnified about 30 diameters.



H. Murayama del.

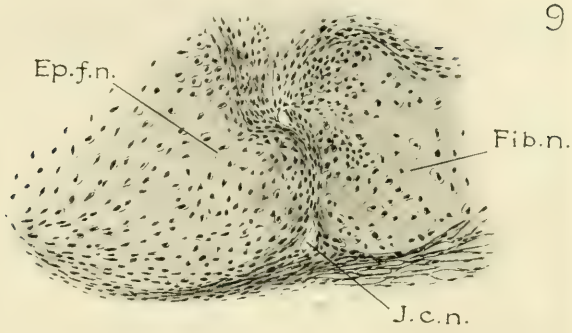
PLATE 3

EXPLANATION OF FIGURES

Figures 9 and 10 are from specimens in which complete amputation of the limb was made at the knee-joint (exarticulation).

9 From a longitudinal section of a limb 41 days after operation. *Ep.f.n.*, new femoral epiphysis; *Fib.n.*, new fibula, proximal end; *J.c.n.*, new joint-cavity forming. Magnified about 100 diameters.

10 From a longitudinal section of a limb 48 days after operation. *C.n.*, new cartilage; *Os.n.*, new bone spreading through the cartilage; *Os.c.*, new cartilage cells transforming into bone-cells; *B.f.*, old bone of the shaft (femur); *M.c.*, portions of the marrow-cavity. Magnified about 420 diameters.



IS THE INFLUENCE OF THYMUS FEEDING UPON DEVELOPMENT, METAMORPHOSIS AND GROWTH DUE TO A SPECIFIC ACTION OF THAT GLAND?

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The experiments on thymus feeding thus far reported in the literature have given results sufficiently different to prevent the formation of a definite idea as to the rôle of the organ in these experiments. This is even true if one has in mind only one of the various groups of animals which have been studied in such experiments. Concerning the Amphibia among which only the larvae of Anura have been studied carefully regarding their reaction to thymus feeding, it seems that most of the experiments showed a retarding influence of the thymus upon development and metamorphosis although some exceptions are reported. With respect to growth however, the results are so lacking in uniformity, that Gudernatsch as well as Romeis who studied the effect of thymus feeding in tadpoles doubted whether the effect produced by this organ was due to a specific action or only to quantitative conditions. Gudernatsch in his experiments on tadpoles noted accelerated growth leading to enormous size; but Romeis obtained completely normal growth in various series of thymus-fed tadpoles and pointed out that the thymus feeding never produces abnormally large animals.

The following experiments which will be reported elsewhere in detail, seem to indicate that the accelerated growth of thymus fed Amphibian larvae is merely the effect of quantitative conditions and not the result of a specific quality of the organ such as a specific growth-stimulating agent. Furthermore, they yielded some very interesting results concerning development and metamorphosis although these are still difficult to explain. Finally they showed that in each thymus-fed larva severe tetany

is produced. The last mentioned phenomenon will be discussed in another article; the effects of thymus upon development, metamorphosis and growth will be outlined briefly in the following pages.

In the experiments to be reported, only larvae of Urodela were studied (*Amblystoma punctatum* and *A. opacum*). The advantage of using Salamander larvae is that the quantity of food given to them can be controlled and measured with exactness, up to a certain degree, which cannot be done if tadpoles are used; and it should be emphasized that in order to avoid errors the possibility of measuring the quantity of food is very desirable and even should be demanded in experiments in which it is suspected that qualitative relations are involved.

1. DEVELOPMENT AND METAMORPHOSIS

Gudernatsch found that the development of tadpoles was delayed if the animals were fed on thymus. Similar results were obtained by Romeis in his first experiments. This led to the inference that thymus contains a substance whose specific property is a retarding effect upon development. Only recently Gudernatsch again published a paper based on this hypothesis.

However, in his work published in 1915 relative to the influence of the glands of internal secretion on Anura larvae, Romeis reports upon two series of experiments, which cannot be explained from the above-mentioned standpoint, and which caused the author himself to doubt whether inhibition of development were indeed a specific function of the thymus. The first series consisted of fairly old larvae of the species *Rana esculenta*, the individuals of which developed in a perfectly normal manner, in spite of being fed with thymus; but in this case it might have been supposed that the thymus feeding had been started too late. In the second series, however, in which larvae of *Rana temporaria* were employed, the thymus feeding was commenced at a very early stage, in spite of which fact the development of the larvae was not delayed. On the contrary, they underwent metamorphosis at an earlier stage than did the larvae fed with

muscle tissue, and before the latter had developed front limbs. This last series of experiments suggests the assumption that the influence exerted by the thymus on development must be dependent on factors not specific for the thymus. The experiments on Salamander larvae now to be described led to similar conclusions.

Both Gudernatsch and Romeis took as index of the rate of development, the growth of the hind and front limbs, the absorption of the tail and the abandonment of the water. The latter phenomenon however, which we will refer to as metamorphosis seems in Salamanders, to be dependent on a mechanism different in many respects from that which controls development, such as the growth of limbs, etc.; for in the first place even under conditions of normal feeding, different individuals show a different stage of development when they leave the water, and secondly the effect of thymus upon development and upon metamorphosis does not seem to be the same in the Salamander larvae examined. Therefore we shall distinguish between development and metamorphosis; growth and differentiation of the limbs, certain changes of the fin and gills not being in direct relation to the abandonment of the water, and the changes of the color pattern of the skin which finally lead to the definite coloration of the skin, will be referred to as development; while the abandonment of the water together with the sudden reduction of the gills to mere stumps and the complete absorption of the fin will be called metamorphosis.

In a group of eight series, O 1916, in which larvae of *Amblystoma opacum* were used, four series were fed with small fragments of earthworms and four series with equal sized pieces of thymus. As will be explained later on, these experiments were conducted with the intention of feeding the respective animals with equal quantities of worms and thymus. So far as development and metamorphosis is concerned, it would seem at least possible, that besides the quality of food, the amount of food may also have some influence upon these two phenomena; but at any rate only if we make the quantities of food alike in the experimental series and the controls, can we be sure that the differences

obtained in both series are the expression of the quality of the food.

The development of the legs and toes was carefully noted, and the development of these organs was seen to occupy a period of from 6 to 9 weeks. As the thymus feeding began as early as the second week, the development of the legs took place under the influence of thymus feeding during 4 to 7 weeks. According to Gudernatsch and Romeis this length of time sufficed in the case of tadpoles to produce the retarding effect upon development of the thymus; but in the case of the Salamander larvae absolutely no retardation could be noted as a consequence of the thymus feeding. Indeed, in those series which as a result of the simultaneous effect of a lowered temperature necessitated a longer period of time in order to attain complete development, a change in the contrary direction could even be noted in the latest stages, the thymus animals attaining complete development of their limbs more quickly than the worm-fed animals.

Thus it can hardly be argued in this connection that feeding had not lasted long enough to produce a result, in view of the fact that in the latest stages of development of the legs when the feeding had lasted a longer period than in the first stage, the opposite result was obtained. This result then indicates that a distinct difference exists between the Anura and Urodela with respect to the effect of thymus-feeding upon development.

The development of the legs in such animals to which instead of equal quantities of food as much food was given as each animal was able to take, was not studied in sufficient detail. In one group of experiments (P 1719) which consisted of *A. punctatum* larvae, development of the legs was recorded during 14 days after the beginning of the feeding; in this group the relation between the thymus-fed animals and the controls was the same as in the above experiments on *A. opacum*.

The differences between the Anura and Urodela become even more accentuated as development proceeds. But a careful distinction must be made between animals fed on equal quantities and those which obtain as much food as they will take.

In the above mentioned group (O. 1916) which consisted of larvae of *Amblystoma opacum* fed with equal quantities, the development of further advanced characteristics of the legs, of the shape of the head, of the gills and of the color of the skin proceeded much more rapidly in the thymus-fed animals than in those fed on worms.

With respect to the development of the gills, the following should be remarked: In larvae of *Amblystoma opacum* fed with worms and kept at an average temperature of 22.6°C . as late as one or more days before metamorphosis the gills attain a stage not only of considerable size, but one in which they are characterized by considerable redness and above all by the fact that they are bent upwards in a crescent shape. The long well developed branches are widely extended and the points of the stem inclined forwards so as to bend over. In worm-fed animals kept at a high temperature (22.6°C .) this condition of the gills was only attained in the 23rd week, but in thymus-fed animals kept at the same temperature as early as the 11th week; in worm-fed animals kept at a low temperature (in average 14.8°C .) only in the 29th week; in thymus-fed larvae kept at the same temperature as early as the 11th week (although in the latter case the gills were less developed than in the case of the high temperature thymus-fed animals).

A similar relationship is observed with respect to the color of the skin. In the case of the warm worm-fed animals the melanophore spots only began to develop in the 13th week, at which time they had already attained very considerable development in the case of the warm thymus-fed animals; in the warm worm-fed individuals the blue-grey pigment did not appear until the 24th week, but in that of the warm thymus-fed animals as early as the 12th week. In the cold worm-fed animals the fusion of the melanophore spots into a uniform black-brown coat only began in the 30th week, and occurred as early as the 13th week in the case of the cold thymus animals; but in the cold worm-fed animals no trace of a silver-grey pigment can be detected after 32 weeks, although this appeared in the cold thymus-fed individuals as early as the 13th week. These differences in the

rate of development are doubtless sufficiently great to indicate distinctly the differences existing between the Anura and Urodela. Under conditions of quantitatively equal feeding (which alone can be taken into consideration in a study of qualitative effects) the feeding of thymus to larvae of *Amblystoma opacum* causes accelerated development.

Nevertheless, the above mentioned experiments become even more clear if the results obtained by them are compared with the result in experiments made by a different method; for the factor to be emphasized is not the time elapsed since the hatching but the size of the animals. The thymus-fed individuals attain the stated conditions of development while much smaller in size than the worm-fed animals. The latter must attain much greater size than the thymus-fed animals, in order to acquire the same degree of development.

In a group of *A. punctatum* (P. 1916) kept at a high temperature, one series was fed with pieces of thymus and another with Tubifex. In both series the animals were allowed to eat according to their inclination as a result of which the worm-fed animals consumed a considerably larger quantity of food than did the thymus-fed animals, and consequently grew much more rapidly. The development of the skin pigmentation also proceeded more quickly than in the case of the thymus animals, although the latter attained these various stages while much smaller in size. At the time no exact drawings were made to show the relationship between the size and stage of development. This will be taken up in a recently initiated experimental series of *A. punctatum* (P. 1917) as yet incomplete, in which the same system of feeding is being maintained as in Group P. 1916. Meanwhile it can already be noted that the worm-fed animals do not develop the yellow network until they have attained the average size of 62.91 mm., the minimum length being 59 mm. The thymus animals, which have only attained an average length of 32.22 mm., with a maximum length of 36 mm., have not yet shown signs of this network. In group P. 1916 of *A. punctatum* in which the worm-fed animals behaved like the worm-fed animals in Group P 1917 regarding the relation between size and de-

velopment of network, the first thymus animal attained the network stage when only 41.3 mm. in length.

We thus see that the time at which the various phases of development are attained varies according to the quantity of food, with the result that sometimes the thymus animals, at other times the worm-fed animals appear to lead. But the constant factor is the size at which the various stages are attained; that is, constant to the extent that the thymus animals always develop more quickly than do the worm animals, if the various stages are referred to the size of the animals. This relationship is directly opposed to that of the *Anura* larvae, for in these animals the thymus-fed individuals must usually attain a considerably greater size than the worm-fed animals in order to arrive at the same degree of development.

Identical relationships as occur in the development are also found in the metamorphosis; but in this case one or more additional factors seem to play a rôle to complicate considerably the phenomena, as we shall see.

Here again we must differentiate between the experiments in which the food was quantitatively equal and those in which each animal was allowed to eat to the point of satiety. But it should be emphasized that only the first method permits of a correct comparison. For when the worm animals feed at will they eat approximately 10 to 20 times the quantity of food that is consumed by the thymus animals when the latter begin to suffer from tetany; as the worm animals also grow much more rapidly as a result, it would not be surprising that they also metamorphose earlier, since we might expect that if a definite size of the animal is indispensable to metamorphosis, metamorphosis will be accelerated if we accelerate growth by some external conditions.

We will now turn our attention again to the group O 1916 of *A. opacum* in which each series was given approximately the same quantity of food. In this group the warm thymus animals were the first to undergo metamorphosis; thus in the warm thymus series (22.6°C.) the first animal underwent metamorphosis in the 13th week, in the warm worm series only in the 24th week; in the cold thymus series (14.8°C.) the first animal left the water

in the 24th week; whereas in the cold worm series no animal has yet undergone metamorphosis (in the 32nd week). Thus, thymus-fed animals are seen to metamorphose earlier than worm-fed animals; that is, provided they receive equal quantities of food.

The relationship of time however becomes inverted if the worm and thymus animals, instead of receiving equal quantities of food are allowed to eat at will. In a group of *A. punctatum* (P 1916) consisting of two series, which had been kept at a high temperature and in which the last-mentioned mode of feeding was adopted, the first animal of the thymus series underwent metamorphosis after five months, whereas the first of the worm series did so after only $3\frac{1}{2}$ month

As in development, so also in metamorphosis, the relationship of time is seen to be inconstant and depends on the amount of food given to the animals. But a constant factor exists in the relationship between size of the animal and metamorphosis. Whatever method of feeding may be adopted, the thymus-fed individuals are always much smaller when they undergo metamorphosis than are the worm-fed ones. In the *Opacum* group (O 1916) consisting of equally fed animals, the warm thymus animals averaged only 47.8 mm. in length at the time that the first individual underwent metamorphosis, whereas in the worm series at the beginning of metamorphosis the average size was 53.5 mm. The same relationship can be observed at a low temperature; the average size of the thymus animals being only 57.5 mm. at the beginning of metamorphosis, whereas the worm animals had not yet begun to metamorphose when their average length was 65.1 mm. The same conditions apply in the above-mentioned *Punctatum* series (P. 1916); the thymus animals begin to metamorphose when their average size is 41.9 mm., but the worm animals only at an average size of 50.0 mm.

As in the case of development, so in metamorphosis the relationships obtaining in *A. opacum* and *punctatum* are exactly the reverse of those found in the *Anura* larvae, for in the former the worm-fed animals must attain a much greater size than the thymus-fed individuals before they can undergo metamorphosis

whereas in the case of the Anura larvae the thymus animals must be larger than the worm animals before metamorphosis can occur.

However, in addition to the facts mentioned above, still another phenomenon must be described which seems to aid greatly our understanding of the relation between development and metamorphosis. If we refer metamorphosis neither to the time which has passed since hatching nor to the size of the animals but to the stage of development of certain structures, metamorphosis does not appear to be accelerated in the thymus animals but rather retarded.

For example, when comparing the warm worm animals with the warm thymus animals of the Opacum group (O 1916) we see that as early as the 11th week the warm thymus animals attained the same stage of development at which the warm worm animals commenced to metamorphose. At this stage, however, a remarkable phenomenon is noted; the warm thymus animals fail to metamorphose while some of their organs continue to develop; the structures of their skin, which are responsible for the development of the color of the skin attain, while the animal is still larval a phase of development reached by the worm animals only some time after metamorphosis has been accomplished. After the warm thymus animals have entered upon the stage characterized by the crescent-shaped gills and the fusion of the melanophore spots, they should, if compared with controls, undergo metamorphosis, but instead they develop the silver-grey pigment and undergo reduction of the size of the fin. Simultaneously (a point to be specially emphasized) they stop growing and become reduced in length, a condition which also occurs in the case of worm animals before metamorphosis. They assume an aspect which on the whole resembles that of a worm-fed animal which had undergone metamorphosis about two weeks previously. As can already be seen, these relationships can be noted much more distinctly in the cold Opacum series; but as the animals of these series have not yet all undergone metamorphosis and the worm animals have not yet begun to metamorphose, we will not describe the phenomena already noted.

Exactly the same phenomenon can be seen in a group of *Punctatum* (Group P 1916) maintained at a high temperature, such as the development of definite characteristics of a metamorphosed animal during the larval stage. In this case the yellow network was separated into yellow spots during the larval stage—a phenomenon which does not occur in the case of the worm animals before they have left the water.

From what has been stated above we can see that even in those animals which metamorphosed first and, in the series of *Opacum* larvae (O 1916) fed with equal quantities, metamorphosed 11 weeks earlier than the worm animals, the process of metamorphosis was disturbed. This becomes much more apparent if for the date at which the first animal underwent metamorphosis we substitute that of the last animal metamorphosed. In that case we obtain the following relationship: In the series of *Opacum* larvae (O 1916) after 32 weeks have passed, 12 per cent of the thymus-fed animals are yet in a larval stage, whereas the worm-fed individuals all had metamorphosed as early as the 29th week. Thus, the period of metamorphosis in the worm series extended only over 5 weeks, whereas in the case of the thymus animals it has already lasted 19 weeks. In the repeatedly mentioned group of *A. punctatum* larvae (P 1916) kept at a high temperature, the last thymus fed animal had not left the larval stage even after 8 months, whereas the last worm-fed animal had metamorphosed after only $5\frac{3}{4}$ months; thus in the worm-fed animals, metamorphosis covered a period of only $2\frac{1}{3}$ months, whereas in the thymus animals it lasted 5 months. In a group of *A. punctatum* (P 1916 C) kept at a low temperature, a worm-fed series comprising individuals which out of a number of 300 larvae had not yet undergone metamorphosis was added to a thymus-fed series which had been under observation for about 5 months. In other words, this worm-fed series consisted of larvae which were abnormally late in undergoing metamorphosis. The first of these worm-fed animals left the water $5\frac{2}{3}$ months after hatching, the last $7\frac{2}{3}$ months after hatching, the period of metamorphosis extending in this series over 2 months. Of the thymus-fed animals the first metamorphosed after $4\frac{1}{3}$ months,

the last (leaving two animals out of consideration) after $6\frac{1}{3}$ months. In the case of these thymus-fed animals the period of metamorphosis lasted 2 months, for instance, not longer than in the case of the worm animals; but 2 of these thymus animals not yet mentioned, behaved very differently from all the other animals. They both remained at a low stage of development, so far as coloring was concerned, and their tails underwent but slight reduction in size. On the other hand, the gills were reduced to short stumps. Although neither of these 2 animals was shedding its skin (which should take place before metamorphosis) at the time of the reduction of the gills, they were taken out of the water and placed in a vessel, the bottom of which was covered with filter paper and just enough water to keep the vessel wet. But neither of the animals showed any further change, until finally $12\frac{2}{3}$ months after hatching one of them shed its skin and its gills became completely atrophied, while at the same time the skin became darker in color although the yellow network failed to develop. The other animal is still in the larval stage, $13\frac{2}{3}$ months after hatching.

We must not fail however to mention that it still appears very doubtful whether this is a direct effect of thymus, for a similar phenomenon was also noted in the case of worm-fed animals, although not to so extreme a degree. Out of approximately 300 worm-fed animals, only 1 individual showed such a condition; after more than 8 months it was still in a larval condition and had not developed a trace of the yellow network. The fin of its tail was but slightly reduced; its gills were more reduced and the animal was still undergoing growth and taking food spontaneously. It was used for the purpose of an operation, in the course of which it died. However, as has been said, at this stage it showed no trace of approaching metamorphosis. From this it seems very doubtful that the delay of metamorphosis in the two last mentioned thymus animals was actually due to the action of thymus and we must exclude them from discussion until the same phenomenon is obtained in a greater number of cases.

2. GROWTH

In one series of experiments (P 1917) for which purpose larvae of *A. punctatum* which had hatched on the same day and were the offspring of the same mother were employed, it was assumed that where there is an unlimited supply of food, the amount spontaneously taken up by each animal is a function of growth, and that growth is not a function of the food quantity. For that reason in these experiments which were carried out at an average temperature of about 22°C., the animals were allowed as much food as they felt inclined to take.

The group consisted of three series. The animals of the first series were given small equal-sized fragments of thymus with a pair of forceps, until each animal was satisfied. They took the pieces easily and owing to the softness of the material had no difficulty in swallowing them. The second series received fragments of earth-worms. Owing to the hardness of this food, however, the animals found great difficulty in swallowing it, and it took several minutes, or even hours for each piece to be swallowed. As they were fed only once a day, these worm animals remained hungry and consequently were soon backward in growth, as compared with the thymus-fed animals. The latter finding coincided with the observations made in the case of the *Anura*; i.e., that the thymus stimulates growth; but it failed to prove a specific influence of thymus, for the reason that the animals which were fed in a normal manner were found to be starving. In a third series the animals were fed with small worms (*Enchytraeus*), which were at first given in small pieces; these worms were thrown into the containers in such large quantities that the animals never lacked food. Besides this, each animal was fed on pieces of earthworms which the fast-growing animals soon took readily and in large quantities. The individuals of this series grew faster from the very outset than did the thymus animals. As the latter did not develop tetany until the 5th week and were in a completely normal condition until the end of the 4th week, we may look upon the result attained up to that time as the pure effect of nutrition. The salamander

larvae failed to show that the thymus had exerted any growth-accelerating influence. On the other hand, the quantity of food given plays an important part in this connection, for the animals react in a highly sensitive manner to relatively slight differences in food quantities. From these experiments it would seem that in the experiments on tadpoles conducted by Gudernatsch and Romeis the factor revealed is not a specifically growth-promoting influence, but that the accelerated growth of the thymus animals should be attributed to the fact that the jaws of the tadpoles, although adequate to supply the body with a quantity of the soft thymus material corresponding to the needs of the organism, were nevertheless not the most appropriate instrument for preparing from the hard beef muscle sufficient nutriment for the purpose of keeping up normal growth. The very fluctuating results which Romeis obtained in his experiments indicate pronounced sensitiveness on the part of the tadpoles to small quantitative differences of food which often completely escape control, rather than the presence in the thymus of a specific growth-promoting influence. It should also be remarked that in the above-mentioned experimental group it was also noted that the animals fed with worms must consume a much greater quantity of earth-worms than the thymus-fed animals consume of thymus in order to grow equally quickly; the supply of earth-worm fragments which the second series consumed was only slightly smaller than that of the first series fed with fragments of thymus. This fact speaks in favor of relatively high nutritive value in the thymus. It should also be taken into consideration that in the fragments of earth-worms a not inconsiderable part of the volume consumed consists of indigestible substance (chitin, earth) which are later eliminated in the feces.

In the preceding order of experimentation it is seen that at the moment that the tetany period begins in the thymus-fed animals we are confronted by an obstacle which prevents any quantitative judgment from being formed; for from this time on the thymus animals are seen to be abnormally placed and the amount of food taken in by them becomes abnormally low. This is all the more disturbing for the reason that it is uncertain whether

under these conditions the quantity of food spontaneously taken is really a function of growth. On the contrary it appears very probable that the reduced amount of food taken must be attributed to disturbances caused in the swallowing apparatus by the convulsions. In such a case the animals would be in a condition of starvation and in contradiction to the idea of the experiment the rate of growth would be the function of the food quantities introduced into the organism. I thought to be able to overcome this obstacle in another group of experiments, in which I proceeded from the fact that when food is present in sufficient quantities equal amounts of food produce an equal rate of growth.

In a group consisting of 4 series (O 1916) for which larvae of *A. opacum* were used, the food was given in small fragments at the point of the forceps in all the series; an attempt was made to make all the pieces of approximately the same size on the same day of feeding. The number of pieces given to each individual animal was noted, and on each feeding day approximately (for the week) the same number of pieces was given, so that all the animals of these 4 series received approximately the same number of pieces, the series comprising one thymus and one worm group at an average temperature of 22.6°C., and one thymus and one worm group at about 14.8°C. An effort was hereby made to distribute a quantitatively equal amount of food among the 4 series; but it must be remarked that this can only be roughly attempted and cannot be exactly carried out. As it can never be known beforehand how much food the animals may need on a given day in order to be satisfied, it would also be quite impossible to weigh the food. But even if this were possible, the distribution of equal quantities according to weight would not lead to the distribution of equal nutritive quantities as a given volume of thymus contains a larger quantity of substances available for metabolism than does the same quantity of fragments of earth-worms; as has been shown in the first experimental group. Although this method is not exact, it has at least furnished an approximate idea as to how important it is to control the quantity of food in such experiments.

Of course the quantity of food to be given each day was always standardized from the series which desired the smallest amount to eat. At the beginning these were the worm series, and of these the cold worm series showed less avidity for food than did the warm worm series. As a result the thymus series at first received less than they would have liked to eat. The reasons for this comparatively small appetite in the worm animals have been specified above when discussing the first experimental group. From the time that the series of warm thymus animals began to undergo metamorphosis, the animals of this series showed the least desire to eat; after that it was the worm animals in general, and the warm worm series in particular which received less than they could have consumed.

It may be emphasized at this point that when this method of distributing quantitatively equal amounts of food is followed, tetany exerts a very slight influence on growth. Sometimes the rate of growth is reduced at such points where the tetany curve reaches its apex, but in other cases, on the contrary it increases or reaches even a maximum when the tetany curve does.

The condition which exerts an influence on growth in comparison with which all other influences are reduced to insignificance, is metamorphosis, as will be apparent from the following description.

During the first few weeks the warm thymus animals are seen to lead in size; next in order come the cold thymus animals, then the warm worm series, and finally the cold worm series. Nevertheless no special importance must be attributed to this relationship, for as has already been stated, given an equal volume of food, the thymus animals probably obtain more nourishment from their pieces of thymus than do the worm animals from an equal quantity of worm fragments. The relationship of size which has just been mentioned lasts until the 10th week, and the acute tetany which has meanwhile set in among the warm thymus animals and reached its climax has failed to influence this relation at all. In the 11th week a pronounced change sets in; at this stage the warm thymus animals are all ready for metamorphosis, the first individuals being 14 days removed from

this step. During this week the curve of the body size of the cold thymus animals, which up to that time occupied the second position, can be seen to cross that of the warm thymus animals. From the time that the first animal of the warm thymus series entered upon metamorphosis, the warm thymus animals completely stopped growing. Their curve, which of course does not include the metamorphosed animals, is soon after crossed by those of the two series of worm animals, and the warm thymus animals remain smallest in size for the rest of the experiment.

The cold thymus series, the first individuals of which underwent metamorphosis in the 24th week, also increase in size only a little from the time of metamorphosis on; but as the first animals of the warm worm series which is most proximate to the cold thymus curve similarly undergo metamorphosis in the 24th week, and also because the curve of the cold thymus animals is higher above that of the warm worm animals than the curve of the warm thymus animals is above the cold thymus animals, the curve of the latter remains the first at the beginning; it is not crossed by that of the warm worm animals until the latter have all metamorphosed. Finally, in the 29th week, together with the curve of the warm worm-fed animals, it is crossed by the curve of the cold worm series, which now occupies the first position. As early as the end of the 29th week the largest animals of the cold worm series have attained a size greater than that of each non-metamorphosed (and of course of each metamorphosed) individual of the three remaining series. As for the present (after the 32nd week) the animals give no sign of impending metamorphosis and continue to grow.

The above-reported circumstance appears to us to be the most instructive with reference to the statement that thymus-fed anuran larvae attain a size by many denoted as abnormally large but stated by Romeis never to exceed normal limits, although sometimes exceeding the size of the muscle-fed animals. If we begin by comparing each of the two thymus series with the corresponding worm series, we see that the same relation exists between them as between muscle and thymus-fed tadpoles, inasmuch as the animals which metamorphose later attain greater

dimensions than do those which first underwent metamorphosis. It can be seen that this is not connected with a specific influence of thymus feeding, from the fact that exactly the same relation exists between the warm and the cold worm series, the warm worm-fed series which first underwent metamorphosis metamorphosing while smaller in size than the cold worm series, and the latter continuing to grow after the former has metamorphosed. While yet in the larval stage the cold worm-fed animals attain a size which when the largest animals of both series are used for comparison, already exceeds that of the largest warm worm-fed larva by 12.5 mm.¹ From another point of view the salamander larvae of those species so far examined show the very opposite characteristics from those possessed by the anuran larvae; for it is not the worm-fed salamander larvae which first undergo metamorphosis but the thymus-fed individuals. Thus, the point to be primarily emphasized is not the greater size ultimately attained by the worm-fed salamanders and thymus-fed tadpoles, for we have seen that this does not depend upon the specific qualities of the thymus, but that it is a general phenomenon peculiar to amphibia and one dependent upon the time at which the animals undergo metamorphosis. The point of importance in both cases—the larvae of *Anura* as well as of *A. opacum* and *A. punctatum* is the circumstance that thymus-feeding produces metamorphosis in the *Anura* only when considerable size has been attained, whereas in the *Urodela*, on the other hand, this occurs while the animal is but small in size.

To summarize, we may make the following statement: The differences in the rate of growth to be noted before metamorphosis are not the result of a specific growth-promoting influence of the thymus; they are based on the circumstance that animals which are better fed grow more quickly. In the experimental group of *A. punctatum* (P 1917) discussed in the preceding section, these

¹Although the cold worm larvae are at the time of writing larger than the largest metamorphosed warm worm animals, we do not here intend to take up the question of this relation; moreover, a comparison of the experiments hitherto conducted in connection with the *Anura* shows this not to be possible, as the respective authors never observed their experimental animals beyond the period of metamorphosis.

individuals are obviously the worm-fed animals of the third series, which are allowed to have as much food as they wish; in the experimental group with *A. opacum* (O 1916) it is the thymus animals which take in a greater quantity of nutritive material through eating thymus. The experiments furthermore show that qualitative influences exerted on the rate of growth would have to be very considerable in order that they can be experimentally tested in the case of amphibia, for in these animals the slightest quantitative differences, such as can hardly be controlled, would bring about very misleading differences in growth.

With respect to the ultimate size attained by the animals, Salamander larvae resemble tadpoles in the fact that under certain conditions the later they metamorphose the greater is their final size; this is not only true for thymus-fed animals in comparison to worm-fed animals, but also for worm-fed animals kept in high temperature in comparison to worm-fed animals kept in low temperature.

The action of thymus on development and metamorphosis may be summarized in the following way:

In animals fed on thymus the development presumably of the organism as a whole but certainly of the legs, gills, shape of the head and color of the skin, is greatly accelerated during the larval period. The thymus-fed animals, therefore, reach the stage at which worm-fed animals are ready for metamorphosis, much more quickly than worm-fed animals. As development at least to some degree may be dependent on growth, on the rate of growth and on size, it is impossible to examine the specific influence upon development of any substance without keeping alike the conditions of growth in both the experimental and control series; such was attempted by admitting an equal amount of food to both series.

When the thymus animals have reached the stage at which worm-fed animals go into metamorphosis, the development of most organs seems to stop, while certain characteristics of the skin continue to develop; the skin of such animals then behaves very similarly to the sex-organs of neotenic larvae, since the skin at least with regard to the structures determining pigmentation,

develops characteristics of a metamorphosed animal, while the animal as a whole still is in a larval stage. At the time when metamorphosis should occur disturbances in the course of development begin to appear evidently due to the suppression of the development of some factor, without which further development is impossible. In most of the animals of a thymus-fed series this factor still develops much earlier than in the controls; but even in these individuals metamorphosis becomes a grave danger to the animals' life. In high temperature some animals die during metamorphosis and those which survive metamorphosis die a relatively short time after metamorphosis. In some individuals the development of the factor necessary for metamorphosis is still more disturbed and becomes delayed in comparison with the controls; at high temperature all individuals in which this is the case die on the day when the gills and the rest of the fin undergo the sudden reduction in size, characteristic of the entrance into metamorphosis. In low temperature they may survive metamorphosis. In low temperature a very small percentage of the thymus-fed animals may remain at a low stage of development and not metamorphose for more than a year; but whether this is due to the action of the thymus diet is not yet certain, as a similar phenomenon was observed in one worm-fed animal of the stock.

It seems that we cannot understand the results reported in thymus feeding experiments if we assume that they are the pure expression of the influence of the thymus substance. The rather great fluctuations reported in individuals of the same species as well as the surprising differences between larvae of *Anura* and *Urodela* when fed on thymus, indicate that quite a number of factors are involved in metamorphosis, some of which were not controlled in the experiments. It is of course clear, that differentiation of the organism is one of these factors; that a certain degree of differentiation is indispensable for metamorphosis, or at least to facilitate it, was shown by Gudernatsch in some recent experiments on the influence of thyroid. That some of the individuals among a thymus-fed series of Salamander larvae metamorphose earlier than the controls may be due in some degree

to the fact that in the thymus-fed Salamander larvae development and differentiation and consequently metamorphosis also depend on the general conditions of growth; the experiments on Salamander larvae reported suggest that rate of growth and size play an important rôle in metamorphosis. The difference noted between Anura and Urodela when fed on thymus can be explained only by assuming a fundamental difference between the organization of these two groups of animals. It will be pointed out in another article that such a difference, namely the absence in the Salamander larvae and the presence in the anuran larvae of the parathyroids, seems to explain why thymus-feeding should develop tetany in Salamander larvae and should not in anuran larvae. It suggests itself that metamorphosis in part must depend on a factor similarly being present in one group but absent in the other group. The development of that factor may be induced primarily by processes occurring in a certain stage of differentiation, but also may be influenced and inhibited or disturbed by thymus diet. The action upon this factor of the thymus may be widely different from that upon developmental processes preceding its development; this is indicated by the fact that development while accelerated during the larval period is on the contrary retarded from the time at which metamorphosis should occur. It is this phenomenon which emphasizes the fact that metamorphosis to some degree must occupy a particular place among the processes of development. In this connection, finally, frequent reports may be remembered according to which thymus causes disturbances of the blood circulation; in metamorphosis of the Amphibians the blood circulation undergoes a fundamental change in the course of which the gills are absorbed, and in Salamanders, the absorption of the gills according to Maurer, is a prerequisite for the formation of the parathyroids. It may be worth while to keep these facts in mind during further studies of the influence exerted upon metamorphosis by the thymus.

Though the effect of thymus feeding on development and metamorphosis is very evident, it appears to the writer that similar effects may be produced by other and purely quantitative exter-

nal conditions, such as temperature and quantity of food and in general by all factors which modify growth, rate of growth, size and velocity of development. No doubt such factors are of great importance in determining at what time, at what size and developmental stage of the animal, metamorphosis will occur. Since the relations between these different factors are very complicated and the number of experiments relative to them is rather small, discussion of these conditions must be postponed.

Finally it should be mentioned that the thymus gland apparently contains all substances which are necessary to build up the substance of an Amphibian organism to maintain the animal growing and to sustain life permanently. This is demonstrated by a number of specimens of *A. punctatum* kept at low temperature which have been fed on thymus since about the 14th day of their life and are now about 14 months old; they are increasing in size.

THE REGENERATION OF TRIANGULAR PIECES OF PLANARIA MACULATA. A STUDY IN POLARITY¹

J. M. D. OLMSTED

FOURTEEN FIGURES

Morgan ('98), in his studies on the regeneration of *Planaria maculata*, describes two types of operation by which he was able to obtain regenerated pieces in which "the long axis of the new head" was "at right angles to the long axis of the original worm." When he cut narrow strips from the side of a planarian, he found that the piece, through contraction, assumed the shape of a crescent, the cut edge forming the concave margin. In certain cases all the new tissue which formed in the concavity of the crescent was used in the production of a head. A similarly shaped worm was formed in several cases when he cut from the side of a planarian a triangle the apex of which lay within the body. Both these methods of cutting, however, produced other pieces, which upon regeneration nearly or quite retained their original polarity. Morgan remarks (p. 373) "The experiments do not show clearly, why, at one time pieces cut from the side give rise to new worms having the long axis in the direction of the original long axis, and at other times at right angles to the original long axis."

Child ('15, p. 165) states that in triangular pieces cut from the side of *Planaria dorotocephala* the regenerated "head often develops nearly or quite in the direction of the transverse axis."

The possibility of producing regenerated planarians whose polarity has apparently been so changed that their chief axis is at right angles to the chief axis of the worm from which they were taken having been demonstrated, at Dr. H. W. Rand's suggestion a more detailed study of the regeneration of such pieces was undertaken, the results of which are given in this paper.

¹ Contributions from the Zoological Laboratory of the Museum of Comparative Zoology at Harvard College, No. 302.

The species of planarian used in these experiments was *Planaria maculata* Leidy, and the specimens were taken from Fresh Pond near Cambridge, Mass. Worms of various sizes, from 12 to 5 millimeters in length, were used. Some specimens, after being brought into the laboratory, were fed on liver until at the time of operation they were of the maximum size. Others, medium and small worms, were kept without food for several weeks. Neither the condition of satiety nor of starvation noticeably influenced regeneration. At one time the mortality of one lot would be greater, at another time, that of the other. In the fed worms, however, it was found best to allow one week to elapse after the last feeding before the operation was performed.

To prepare the planarians for operation, they were narcotized in a 0.1 per cent solution of chloretone until they ceased to move. Cuts were then made with a sharp scalpel, care being taken to have the cut edges as nearly straight as possible. Triangular pieces were taken from all regions of the body, each triangle having for one of its sides a portion of the original uncut right or left margin of the worm, and, for the other two sides, cut edges which intersected near the original median axis of the worm (fig. 2a, 5a, 7a). The two cut edges, intersecting at a point which I shall refer to as the vertex of the triangle, are distinguished in the following account as the anterior and posterior edges.

It was only towards the end of experimentation that the importance of fairly exact measurements of the lengths of the cut and uncut edges, the angle where the cut edges meet, the distance of the vertex of this angle from the median axis of the worm from which the piece is taken, and the size of the piece, was realized. In the earlier part of the work, no camera drawings were made until the day following the operation. Because of the decided contraction of the pieces at this time and the consequent distortion of their original shape, it was possible to estimate only rather roughly their original measurements. Later in the work, however, camera drawings were made immediately after operation while the pieces were still in chloretone, the very slight contraction in this condition being negligible; a second drawing of each piece was made on the day following, when they

were in the contracted state. The drawings made while the pieces were still in chloretone formed the basis for classification into groups, according to the relative lengths of the cut edges, the size of the angle at the vertex, etcetera. The drawings made on the day after the operation during the earlier experiments were compared with those of the later work and each of the earlier ones was placed in that group which it most resembled. One may fairly assume that pieces which resemble one another on the day after operation would also have been similar immediately after the operation. Thus it was possible to estimate with some degree of accuracy the measurements which the triangular pieces in the earlier work had immediately after operation. In the following account it was thought best, however, to enumerate the cases separately; hence the earlier experiments, in which the original measurements are estimated merely, are referred to as Series I, whereas the later ones, in which the pieces were drawn while still in chloretone, are designated as Series II.

The mortality of such triangular pieces is very great. Less than one-fifth of them survive the operation and regenerate. Pieces taken from the region of the pharynx (fig. 5a) had the greatest vitality, though regeneration of pieces from other regions of the body, if accomplished, proceeded along exactly the same lines as in the pieces from near the pharynx. Bardeen ('03) found that in *Planaria maculata* he could more frequently obtain double-headed worms from cross-pieces when they were taken from the pharyngeal region than when from any other region of the body. Morgan ('04) was also more successful in getting pieces from this same region to regenerate, but he remarks, "Whether this is only because shorter pieces are more easily obtained here, or because the very short pieces from this region survive the operation, remains an open question." The latter explanation seems to be the true one, since in many cases in my experiments the same sized pieces were taken from all regions of the body and only those from near the pharynx survived.

When, in the operation of cutting, the epidermal layer is broken, a great mass of loose parenchyma cells flows out from the wound, and if the two cuts form a very acute angle, the

projecting point on the triangular piece becomes rounded off by loss of material (cf. Morgan, '98, p. 393). Immediately after the operation there is always a very slight contraction of the cut edges, even though the piece is still immersed in chloretone. This, no doubt, is due to the direct stimulation of the muscle fibers. As soon as the effect of the narcotic is gone, the piece contracts greatly, often assuming the form of a hollow cone, the apex of which lies approximately at the center of the dorsal surface of the piece. Epithelial cells soon cover the wound (Lang, '12, p. 272), and after twenty-four hours new white tissue can be seen along the cut edges. This new material is never evenly distributed along the cut edges, but (figs. 2b, 5c, 6c) a greater amount of it appears near the center of the anterior edge, a less amount along the posterior edge, and very little at the vertex (cf. Morgan, '98, p. 378). A further noticeable feature is the lengthening of the posterior side (figs. 5b, 6c, 7c). Even though the two cut edges have the same length immediately after cutting, a few days later the posterior edge is almost invariably the longer. These phenomena indicate that there is a tendency on the part of the triangular piece to retain its original polarity, since the regeneration of a head demands more material than that of a tail, and the lengthening of the posterior edge would more quickly restore the normal planarian form.

Several factors influence the subsequent history of these triangular pieces: (1) size of the piece, and, closely related to this, (2) the angle between the cut edges, (3) the position of the 'vertex' with reference to the original median axis, and (4) the relative lengths of the anterior and posterior cut edges.

The following four rules seem to be obeyed in the regeneration of these triangular pieces.

A. If the vertex of the triangular piece lies beyond the median axis of the worm from which it is taken, the new worm which is formed by the regeneration of this triangle retains the polarity of the original worm, for instance, a new head appears at the anterior cut edge, a new tail at the posterior cut edge, and, near the old median axis, a pharynx, which points toward the tip of the new tail (fig. 1). At first the regenerating worm is cres-

cent shaped, but as regeneration proceeds, it straightens out, until a perfectly bilaterally symmetrical planarian is produced. The number of cases in Series I illustrating this type was 6, in Series II, 10.

This retention of the original polarity occurs even if the angle between the cut edges is quite small, for example 55° , and seems also to be independent of the size of the triangular piece. Sections of the early stages of regeneration of such pieces show that portions of both the original lateral nerve cords are present, and that the new, regenerated nerve cords become linked up with the old to form, eventually, an unbroken loop, the new brain



Fig. 1 Triangular piece from left side of worm, 6 days after operation. Drawing made by reconstruction from serial sections. The broken line represents the boundary between old and new tissue, the new being to the right. The old nerve cords are stippled, the new brain crosshatched. One eye and the pharynx are present.

lying at the bend of the loop. Figure 1 illustrates a stage in which the nerve cord on the left side of the piece (note that the piece was taken from the left side of a worm) and the brain have been completed, but in which the brain has not yet established connection with the portion of the original right nerve cord.

B. If the vertex lies at or near the old median axis, and the angle at the vertex is greater than 90° , the regenerated worm retains its original polarity, unless the piece is from a very small worm. In the latter case (figs. 2 to 4) there appears to be an attempt to return to the old polarity; but lack of sufficient

material, perhaps, prevents the complete success of this effort. Under all circumstances throughout my experiments, with very few exceptions, a new head was formed. It would seem, then, that the tendency to produce a head is so strong that the regeneration of other structures and the attainment of the typical

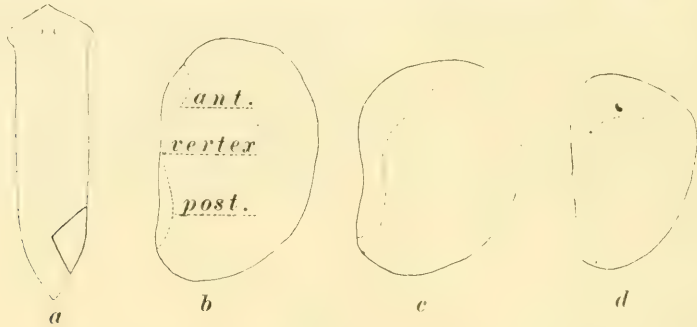


Fig. 2 * Stages of regeneration of a small triangle whose angle at the vertex was greater than 90° . *a*, Whole worm showing region from which piece was taken. *b*, 3 days after operation. All tissue to left of broken line is new. *c*, 5 days after operation. *d*, 11 days after operation. No tail is formed, yet the head is fairly near the anterior end of the piece.

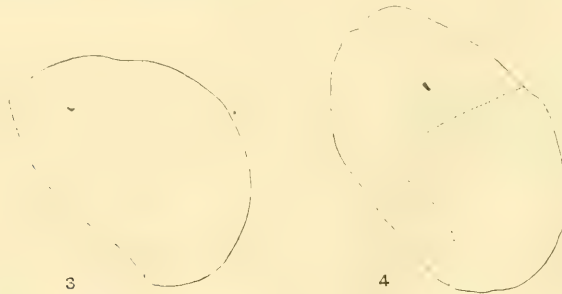


Fig. 3 Triangle from right side of a small worm, angle at vertex greater than 90° . 7 days after operation.

Fig. 4 Similar to 3, but from a larger worm, therefore head almost normal in position.

planarian form is dependent upon the amount of material available after the accomplishment of that object. The number of cases coming under this group was, in Series I, 10, in Series II, 3.

C. If, when the vertex lies at or near the old median axis, and the angle at the vertex is 90° or less, the two cut edges are

decidedly unequal in length, the regenerated worm retains its old polarity. This is true whether the anterior or posterior cut edge is the longer, and has occurred in specimens where the ratio of the lengths of the cut edges was as low as 9:7. The number of cases in which the anterior edge was the longer was, in Series I, 5, in Series II, 2; in which the posterior side was the longer, Series I, 7, Series II, 5.

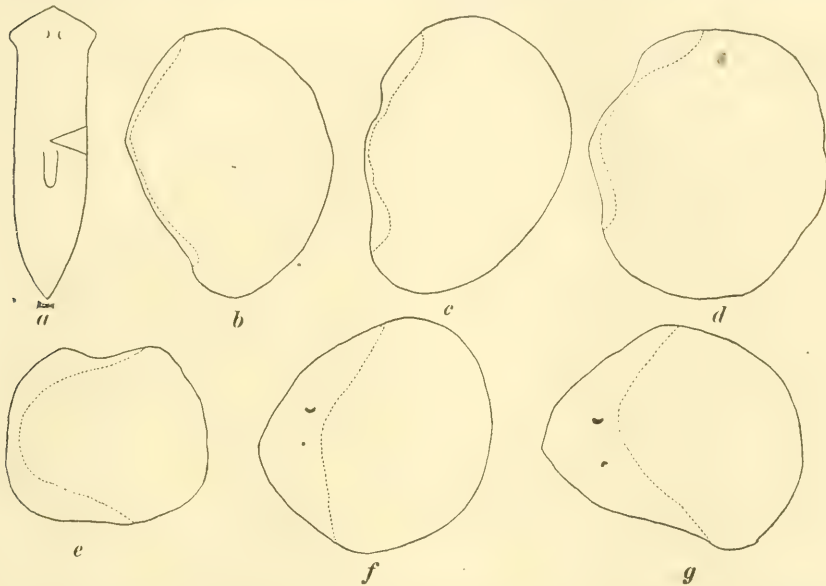


Fig. 5 Stages of regeneration of a triangular piece of worm cut so as to fulfill the conditions for Group D; *a*, showing the region from which the piece was taken; *b*, 1 day after operation; *c*, 2 days; *d*, 3 days; *e*, 5 days; *f*, 7 days; *g*, 10 days.

D. If, when the vertex lies at or near the old median axis, and the angle at the vertex is 90° or less, the cut edges are equal in length, then the piece regenerates a head which is at right angles to the original median axis (figs. 5 to 10); if a pharynx develops, which very rarely happens (two cases only), this organ appears between the eyes and points toward the tip of the new head (fig. 7*e*, 9). The number of cases illustrating Group D was, in Series I, 15, in Series II, 5. In the regeneration of all other types of triangular pieces, the size of the piece influences the

final regenerated form, but several specimens which fulfilled the conditions of Group D and had heads at right angles to the old median axis were larger than those which did not fulfill these conditions and retained their old polarity.

Such a regenerated worm lives for days or even weeks without change, except a diminution in size, until it disintegrates. It rarely moves about in its dish, but when disturbed it moves rap-

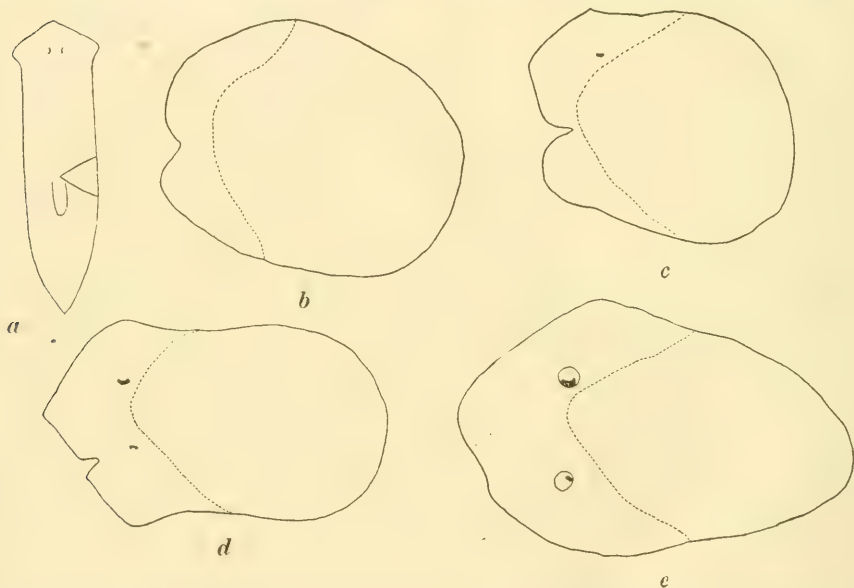


Fig. 6 Case similar to that of figure 5; *a*, showing region from which the piece was taken; *b*, 3 days after operation; *c*, 5 days; *d*, 7 days; *e*, 14 days.

idly in the direction in which the head points, or in wide curves with the original posterior edge directed toward the center of revolution.

During the first two or three days of the regeneration of these triangular pieces whose heads develop at right angles to the old long axis, accumulation of new material occurs along both anterior and posterior edges, but the amount along the anterior edge is usually the greater (figs. 5*d*, 6*c*, 7*b*). Morgan ('98), in speaking of the regeneration of right-angled triangular pieces, says,

"Although the amount of old tissue at the anterior end is relatively little, yet the growth of the new material that forms in this region is greater than that of the material at the side immediately behind the head and along the side of the piece. More material is formed where the head is to appear than behind that region." He thinks it probable that this is due to greater cell growth at the anterior end rather than to a migration of cells into this region.

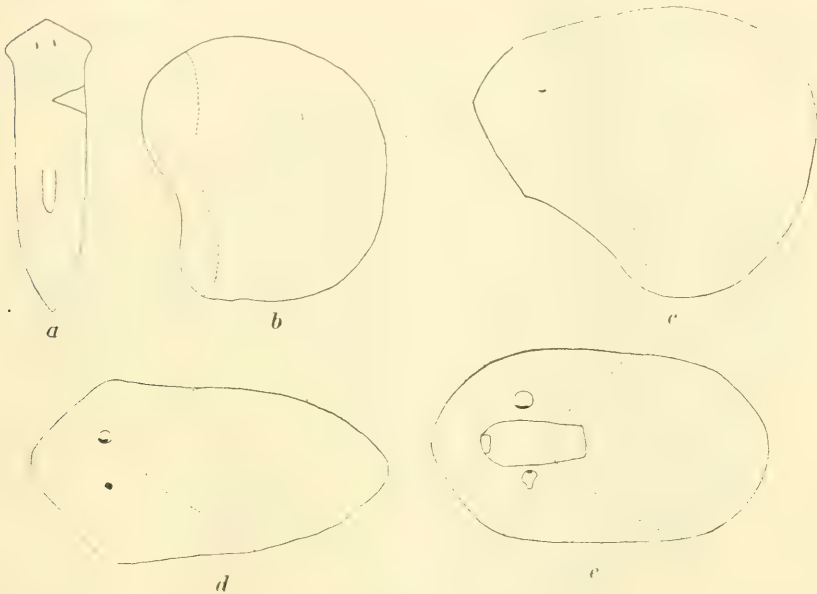


Fig. 7 Case similar to that of figure 5; *b*, after 3 days; *c*, 5 days; *d*, 9 days; *e*, 19 days.

One could suppose from an examination of many of the pieces in the earlier stages of regeneration (figs. 5d, 6b, 7b) that a new head would be formed along the anterior, and a tail along the posterior edge, just as in the case of pieces which are cut obliquely, since the accumulations of material at the centers of the anterior and posterior edges are so distinct from each other. This, however, does not take place. The indentation which often marks the boundary between anterior and posterior accumulations of new material becomes less and less pronounced until it is

obliterated and all the new material goes to form the head (figs. 6, 7). Without exception, the first eye to make its appearance is the one which lies nearer the anterior boundary of the uncut surface (figs. 6c, 7c). This is likewise the rule in the regeneration of heads along oblique cuts (Rand and Boyden, '13). The other eye develops later and remains smaller for many days, or even until the worm disintegrates (figs. 5g, 6e, 7e, 8, 9).

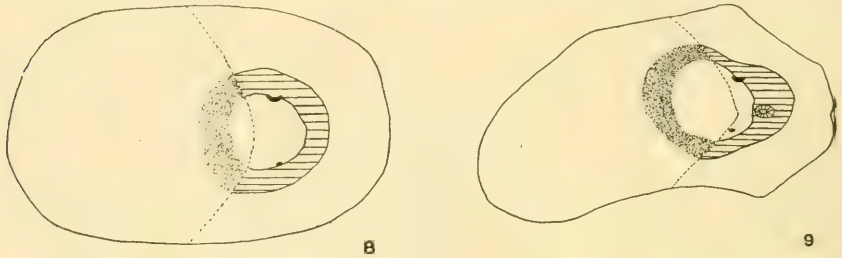


Fig. 8 The most nearly symmetrical of all pieces obtained. Piece taken from left side of worm near pharynx. Drawn in fixing fluid on 13th day after operation. Brain and nerve cord (cross hatched) filled in from serial sections.

Fig. 9 Case similar to that of figure 8, 15 days after operation. New pharynx, and unequally developed eyes.



Fig. 10 *a* A camera drawing of a frontal section whose photomicrograph is given in figure 11, showing position of nervous system. 10 *b*. Whole animal reconstructed from serial sections and from last drawing of piece while alive. New tissue at the left of broken line.

One of the striking characteristics of these triangular pieces is the condition of the nervous system. Frontal sections of six such pieces, after regeneration of one or both eyes, showed that the two new nerve cords are joined to the ends (anterior and posterior) of the remnant of the old nerve cord (figs. 8 to 11).

The nervous tissue, therefore, has the form of a circular or oval ring. Miss Keiller ('10) found a somewhat similar condition in the nervous system of heteromorphic heads of *Planaria simplicissima*, the old nervous tissue in this case being the brain. She

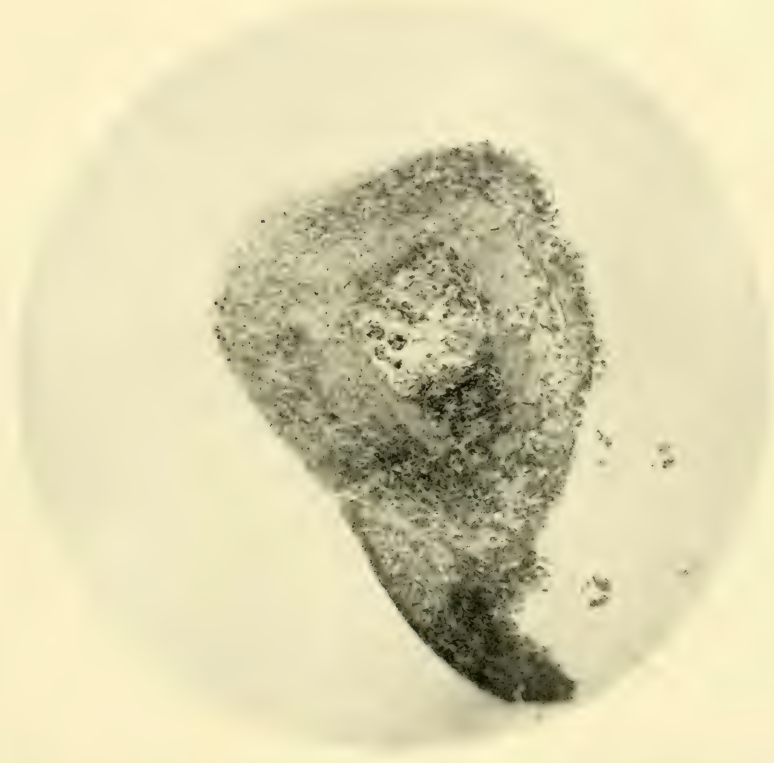


Fig. 11 Photomicrograph of a frontal section of a triangular piece of Group D, showing nervous system in form of a ring surrounding tissue from the digestive organ (cf. fig. 10 *a* and *b*).

states, "The new brain is almost an exact counterpart of the old; and the two together form an almost circular structure."

Lang ('12) and others (Flexner, '98; Stevens, '01; Shultz, '02), as opposed to Lehnert ('91) and Bardeen ('01), claim that in the regeneration of planarians the new nerve cord arises *de novo* in the new tissue at the ends of the old nerve cords, and never

as a proliferation from the old nerve cord itself. All the sections I have examined tend to support this view, since the old nerve cord presents the same appearance in all stages of regeneration, and the new nervous material is absolutely distinct from the old. If this view is correct, the fact that one eye appears earlier than the other, and that this eye is on the same side of the body (in these triangular pieces) as the old nerve cord, may be explained by supposing that the differentiation of nervous tissue is a progressive process, which begins at the anterior end of the fragment of old nerve cord and completes the nerve cord anteriorly on that side first; then, after the formation of the portion of the brain on the same side, the portion of the opposite side is produced, and after that, the nerve cord of the opposite side, which finally joins with the posterior end of the old nerve cord. The side upon which the brain is first differentiated would, of course, be the first to develop an eye.

Morgan ('98) was unable to find a pharynx in any of his regenerated triangular pieces. In one case in my experiments (fig. 7) a pharynx was fully developed. It was formed in the old tissue as in the ordinary course of regeneration, but its position was anomalous. The opening through which it projected lay on the dorsal surface between the two eyes, and the pharynx itself was directed forwards in the long axis of the regenerated worm. Often when disturbed the animal thrust out the pharynx beyond the head and then drew it back. An attempt to feed this animal with liver to prolong its life was unsuccessful. No food could be seen to enter the pharynx, though it executed, rather feebly, the feeding movements. The worm began to disintegrate on the 21st day, and an attempt to fix and section it was made. In a second specimen no pharynx was visible externally, but on the 15th day, while the worm was still vigorous, it was killed and fixed, and later sectioned. The sections revealed that such an organ had begun to develop between the eyes (fig. 9). The position of the pharynx is not quite so extraordinary as it at first seems. Had the piece regenerated a head along the anterior and a tail along the posterior cut, the position of the pharynx would have been nearly that which is

normal for the regeneration from such oblique surfaces, for instance, approximately mid-way between the potential head and tail. But, since the new material along the posterior cut fused with that along the anterior cut to form a head, the pharynx was forced to take a direction practically perpendicular to the original long axis of the worms, instead of the usual angle of about 60° to the old median axis—the position which obtains during the early stages of regeneration of triangular pieces which retain their original polarity.

Two other anomalous specimens must be mentioned. Two pieces cut from the pharyngeal region of different worms failed to produce heads, though they lived some 20 days and each regenerated a pharynx. Morgan ('98) found that in an excessively acute triangular piece, whose acute angle was at the most anterior part of the piece, the contraction of the tissue after cutting caused this sharp point to bend over and fuse with the side, thus preventing regeneration at the most anterior portion of the piece. Similar headless forms were obtained from cross-pieces in which a notch was made on the anterior surface, the fusing together of the edges of this notch preventing regeneration of a head. Child ('15) produced forms of varying degrees of headlessness by allowing pieces to regenerate in various solutions which retarded metabolism. The two headless specimens in my experiments did not arise in any of these ways, but both developed from triangular pieces of the same size and appearance as those which regenerated heads, and were treated in exactly the same manner as the others. The history of one of these pieces is shown in figure 12. A similar headless specimen regenerated in one instance from a piece cut from a planarian as in figure 13. These three headless worms were very sluggish, and although each had a pharynx, attempts to feed them were unsuccessful.

Morgan ('98) and Child ('15) have both commented on possible causes for the peculiar regeneration of the triangular pieces which develop heads at right angles to the long axis of the worm from which they are taken. Morgan ('98, p. 374) says, "The

results show that the worms with axes at right angles to the original axes are most often present when long narrow strips are cut from the side. Pieces of this kind show a marked tendency to become crescent shaped. . . . The new material is formed in the concavity of the crescent, and it is all used up in the

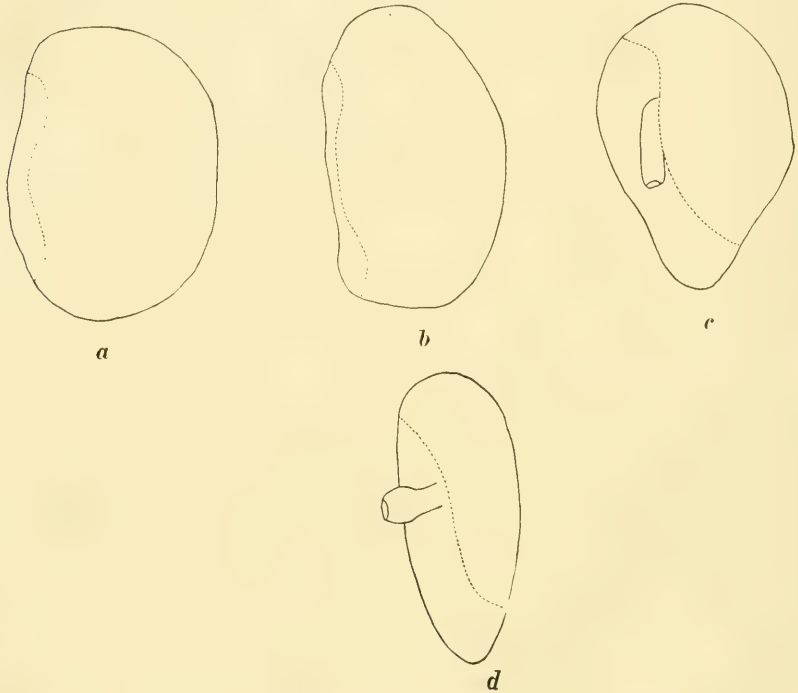


Fig. 12 Stages in the headless regeneration of a piece from right side of worm. *a*, 5 days after operation; *b*, 8 days; *c*, 14 days; *d*, 20 days; *d* was drawn while the piece was in chloretone, hence the protrusion of the pharynx.

formation of the new head. There is no material left over for the formation of the other parts." He adds, "This is not, of course, an explanation of the phenomenon, but it is only a restatement of the facts." Again he remarks in regard to cross-pieces ('01, p. 46). "Since the tendency to produce a head approaching the maximum size is stronger than the tendency to produce as much of the missing anterior end, all the new material goes into the new head."

It is very evident that such a head-forming tendency is present and that, with rare exceptions, it is always in operation. This tendency always to produce a head, together with the small amount of new material available, are two most potent factors in the regeneration of small pieces of planarian tissue of any shape. But my experiments show that there are still other factors which must be taken into account, since some small triangular pieces retain their original polarity and develop both a pharynx and a tail in addition to a head, while others, which may actually be larger, regenerate heads, and heads only, at

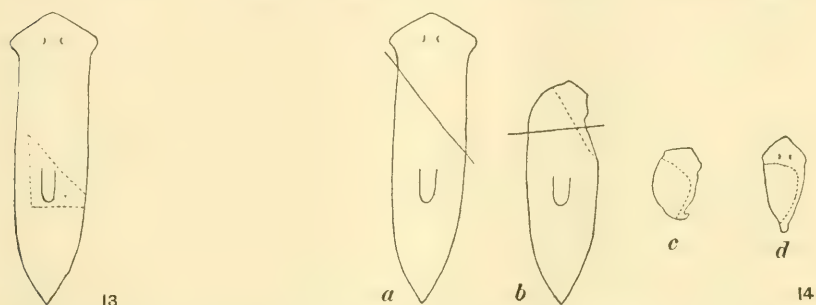


Fig. 13 The piece within the broken lines, which represent the cuts made, regenerated no head.

Fig. 14 *a*, First cut indicated by oblique unbroken line. *b*, Head regenerating after first cut. Second cut indicated by unbroken line. Broken line indicates limit between old and new tissue; *c*, 4 days after second cut; *d*, final regenerated form. Original polarity restored.

right angles to the old axis. These other factors, brought out in the experimental work, are (1) the position of the vertex of the triangle. (2) the size of the angle at the vertex, and (3) the ratio of the lengths of the anterior and posterior cut edges. Child's ('15, p. 165) explanation for the position of the heads in these triangular pieces is, "In such pieces there is little difference in metabolic rate between apical and basal cut surfaces, and the cuts are not sufficiently oblique so that the higher level in the major gradient of the lateral as compared with the median region of the cut surface overbalances its lower level in the transverse gradient. Consequently the median regions of both cut surfaces represent the region of highest rate or irritability and therefore

become the head-forming region." The factors which were enumerated above can all be expressed in terms of Child's axial gradient theory, though one feels that polarity is a more deep-seated phenomenon than differences in metabolic rates.

Morgan ('98, p. 374) makes a statement with reference to long narrow side pieces which is very suggestive. "It may be claimed that the small, single-headed worms with the long axis at right angles to the original long axis, do not really stand in this relation to the old part, but the head is at the anterior end of the piece, and being fused with the entire inner edge of the crescent is unable to swing around later into position." It has usually been considered that in regeneration from oblique cuts the polarity of the new material undergoes change, since the axis connecting the new head and tail at first lies at right angles to the cut edges, but later comes into line with the old chief axis. However, the fact that the geometrical median axis of the regenerating worm does not pass through the new head and tail does not necessarily prove that the structural axis of these new parts is discontinuous with the axis of the old part, nor does it prove that there is any essential difference in the polarity of old and new parts. Sections show that the new systems of the worm, especially the nervous and digestive systems, are perfectly continuous with the old systems; the median axis, instead of being a straight line, as in the fully regenerated worm, is, in the early stages of regeneration, a curved line, which may even approach a semicircle. At Dr. Rand's suggestion, experiments devised to test the polarity of old and new tissues in regeneration were carried out. An oblique cut anterior to the pharynx, and quite across the body, was made on thirty planarians (fig. 14a). As soon as the head appeared (in its usual position at right angles to the cut), a second cut was made as in figure 14b. The resulting piece of planarian was thus given a form which closely resembled that of the typical planarian, and one which was almost geometrically symmetrical, but whose geometrical long axis did not coincide with its original long axis. Had the polarity of the new head along the obliquely cut edge been truly different from that of the rest of the worm, one would expect that under

the conditions of the experiment the dominance of the head-forming material would assert itself, as it appears to do in the regeneration of the small triangular pieces, with the result that the piece would retain its artificially made form, the new head remaining where it was, the point of tissue at the opposite end serving for a tail, morphallaxis—which is pronounced in this species (Morgan, '01, p. 48)—taking place in the old tissue to make the morphological long axis coincide with the artificially established geometrical long axis. No case was found in which the piece retained this form. The head swung round so as to come into line with the old axis, and at the posterior end appeared a tail, which in some cases was so curved that it actually pointed anteriorly, but eventually straightened out. If the piece was so small that no tail regenerated, enough material appeared along the posterior edge to make a perfectly symmetrical worm with its original polarity restored. In no case was the polarity permanently changed, but the structural long axis of the regenerating worm did not coincide with its geometrical long axis until after regeneration was complete (fig. 14d).

In the regeneration of all triangular pieces there was evidence to show that there was a similar tendency on the part of the piece to retain its original polarity. The prevention of its full expression was probably due chiefly to lack of new material. This tendency was indicated in the following ways: (1) by the production, in the early stages of regeneration, of a mass of new material along the posterior cut distinct from that along the anterior cut; (2) by the greater quantity of new material along the anterior cut; (3) by the formation of the head nearer the anterior end of the piece; (4) by the appearance of the more anterior eye first, even in those pieces in which the head was clearly at right angles to the old axis; and (5) by the fact that not a single specimen whose head did not come into line with the old axis ever regenerated a tail. The failure to produce a tail is very significant. If the polarity of a triangular piece with a head at right angles to the original long axis is such that the new head is truly at the anterior end, and the old uncut (right or left) margin is truly the posterior end, then either a portion

of the original lateral margin must serve as a tail, or a tail is unable to appear on this 'posterior' end because there is no new tissue at this region to form one. The former of these two possibilities was shown by the experiments illustrated in figure 14 not to have been realized under seemingly favorable conditions. The planarian did not suffer a lateral margin to serve as a tail, but produced a new tail, or at least added enough new material to make the old lateral margin again lateral in the new worm. To test the second possibility, the 'posterior' ends of four of these triangular pieces whose heads had already been developed and polarity established, were either cut off or so wounded that new tissue appeared there. In all four cases the wound healed over with only enough new material to repair the injury. No sign of a projection which could be taken for a tail was to be seen. In these triangular pieces the new material which at first appears along the original posterior edge may be potentially tail-forming substance, but on account of the overwhelming tendency toward the production of a head, this material moves anteriorly, becomes a part of the head, and all possibility of regenerating a tail is lost.

There is a possibility that the peculiar condition of the nervous system in these triangular pieces may inhibit the regeneration of a tail. Had regeneration proceeded along the same lines as in large triangles whose angle at the vertex is more than 90° , a tail would have been formed in the new tissue just posterior to the end of the old nerve cord (or its prolongation). In this region the two nerve cords, the old and the new, run parallel to each other, as do the two old nerve cords in figure 1. But what actually happens is that the old and new nerve cords in such pieces never lie side by side, but become joined end to end. Figures 8 to 11 show the nervous system of these pieces so completed that there is no place in the scheme for a tail. It is more probable, however, that the factors which determine that the nervous system shall be an unbroken ring are the very ones which also determine that there shall be no regeneration of a tail. The influence of the nervous system on regeneration is, however, an unsettled question.

These expressions of the tendency of all triangular pieces to retain their original polarity, together with the fact that slight deviations from certain definite positions of the cuts always result in complete failure to displace the original polarity, lead one to suspect that, even in those cases in which the head appears to be at right angles to the original long axis, the original polarity has not been fundamentally changed.

SUMMARY

1. Triangular pieces cut from the side of *Planaria maculata*, as in figure 2a, regenerate heads at right angles to the original long axis of the worm from which they were taken only when the following conditions are fulfilled: (a) the point of the intersection of the two cut edges must lie at or near the old median axis of the worm; (b) the angle between the cut edges must be 90° or less; (c) the cut edges must be of the same length.

2. Under all other conditions the original polarity is most evidently unchanged. If the piece is small, it may regenerate a head only, but the position of this head shows the tendency to retain the old polarity.

3. In triangular pieces whose heads are at right angles to the original long axis the first eye to appear is the one which lies nearer the anterior end of the uncut edge.

4. The nervous system of such a regenerated triangular piece is an unbroken ring.

5. If a pharynx is developed, it appears between the eyes and points toward the tip of the new head.

6. A head-bearing piece of *Planaria maculata* whose geometrical long axis, artificially established, does not coincide with its original morphological axis, will not retain the form in which it was cut, but regenerates with its original polarity unchanged.

7. All the data of these experiments point to the possibility that polarity, even in the most extreme cases, has not been fundamentally changed.

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THE OLFACTORY REACTIONS AND ORGANS OF THE
MARINE SNAILS ALECTRION OBSOLETA (SAY)
AND BUSYCON CANALICULATUM (LINN.)

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I. INTRODUCTION

It has long been recognized by fishermen and naturalists that many species of marine carnivorous snails are conspicuously successful in finding food. To the former they may be a source of considerable annoyance through their habits of entering lobster pots, eating fish entangled in nets and feeding upon bivalve mollusks, sometimes doing so much damage that like the whelk and oyster drill they become serious pests. The extensive

literature devoted to mollusks contains numerous references to these gasteropods congregating in great numbers about dead animals, and several observers have described buried snails coming out of the sand when food was placed in the water near them. This ability of the carnivorous snails to find food has quite generally been attributed to a well developed sense of smell, but very little definite information concerning their olfactory reactions and organs appears to have been obtained by experimental methods. Most recent writers in discussing the sense of smell in this group refer to the work of Nagel ('94), whose studies on marine gasteropods however were evidently limited, and who, by stimulating them with strong or irritating substances, used stimuli which were inappropriate for calling forth olfactory reactions, at least so they could be distinguished from other types of chemical responses. Accordingly an experimental investigation of olfaction in two species of snails was undertaken in view of determining particularly the sensitiveness of the animals and the characteristics of their responses to olfactory stimuli, whether they are directed to food or find it by random movements, and the location of the receptors concerned in the reactions.

A part of the work was carried on at the United States Fisheries Biological Station at Woods Hole, and I wish to express my gratitude to the resident Director, Dr. P. H. Mitchell, for many favors received during my stay.

II. EXPERIMENTS

ALECTRION OBSOLETA

An investigation of olfaction in marine gasteropods was begun by a study of the food reactions of the mud snail, *Alectrion obsoleta* (Say), formerly included under the genus *Nassa* or *Ilyanassa*. It is a rather small species, the expended foot measuring from fifteen to eighteen millimeters in length, and about ten millimeters in width in its broadest part. The anterior border of the foot is extended on both sides so as to produce recurved processes, approximately one millimeter long,

provided with sharply pointed tips. The slender tapering tentacles, about five millimeters long, are borne on stout processes projecting in an antero-lateral direction three millimeters from the head, and the eyes are situated at the base of the tentacles on the outer side. The siphon, which runs forward above the head, represents a prolongation of the mantle. The ventral wall is split its entire length, but when the edges are brought together a tube is formed, open at the tip, through which water passes to the gill within the mantle chamber. The organ protrudes from ten to fourteen millimeters beyond the anterior border of the shell, and can be swung about in various directions.

Alectrion occurs commonly on tide flats, where it is a thorough scavenger, feeding on a variety of food substances, but often to be seen collected in great numbers on some dead animal such as a fish or a crustacean. Dimon ('05) writes of its food as follows:

Among the various food materials upon which *Nassa* was seen to feed were hen's egg shells, dead hermit crabs and *Squilla*, live *Nereis*, ulva, the alga that grows upon the shell of *Nassa* itself, the thick black mud of the inner harbor, and the alga that gathers on the glass of aquaria. . . . *Nassa* did not usually attack a live uninjured clam, though I have seen the snails collected at the edge of the mantle of a clam that was apparently alive, devouring it and preventing the shell from shutting by pushing themselves into the opening between the valves.

Belding ('10) has shown that *Alectrion* occasionally feeds upon the living scallop, gaining access to the soft parts of the mollusk by entering between the open valves.

1. *Reactions to olfactory stimuli*

That an olfactory sense plays an important part in the discovery of food by *Alectrion* seemed probable. Dimon ('05) reports finding a *Squilla* about six inches in length

on the mud, with ninety-eight mud-snails crowding about it. It was then taken away from these and put into a pool about eighteen inches in diameter, which was full of quiet mud-snails. These snails immediately became active, but without moving definitely toward the *Squilla*. When one happened to reach it, it stopped and began to

eat, so that in a short time they were once more gathered thickly about the Squilla. The same test was repeated in another pool, with the same aimless wandering and gradual collecting of the snails about the Squilla. In the laboratory the Squilla was put into an agate pan about sixteen inches square, in which were thirty-five mud-snails. The effect was the same as in the pools out of doors. The snails moved about actively at first, and in the course of twenty minutes, twenty of them had collected on the Squilla and the others had quieted down.

The Squilla was large and had probably been dead for some time, so that its odor or taste was quickly diffused through the pool. Towards smaller, live creatures, such as *Mya arenaria* with a cracked shell, the response of *Nassa* was less rapid, and was not at all definite, unless the snail came very near the clam. The definiteness and promptness of response seemed to vary somewhat with the nearness of the animal to the stimulus, and also with the individual snail.

This description gives the impression that the food was scented by the snails, but that its discovery was perhaps largely a matter of chance. In the hope of gaining more detailed information on the behavior of *Alectrion* in the presence of food, the following experiment was tried:

A shallow rectangular glass dish, measuring approximately thirty-eight by twenty-three centimeters, was filled with sea water. At one end of the dish was placed a ball of cheese cloth about three centimeters in diameter, and at the opposite end a cheese cloth bag of the same size containing fresh fish (*Fundulus*) meat. Both packets were weighted with a pebble to hold them in position. Ten snails were then placed along a line midway between the two packets and their behavior noted for one hour.

That the snails scented the fish when some distance from the baited bag was soon made evident. The bottom of the dish was slightly concave at the margins, so that the fish juice slowly drained toward the two corners nearer the baited bag. The snails which moved into the region of the juice extended their proboscides, and worked them over the surface of the glass. This proboscis reaction, which later was carefully studied and found to be characteristic of snails stimulated with dilute food materials, often beginning when the animal was several centimeters away from the packet, was particularly marked where the juice drained toward the corners of the dish, and sometimes occurred in the corners, which were about ten centimeters from

the fish meat. The proboscis was extended farther when the snail approached the source of the stimulating material closely, and two or three of them climbed upon the bag in their attempts to secure the food. Sometimes the snail's siphon was brought within one or two millimeters of the bag before the proboscis was protruded. This was especially true when the bag was approached from the center of the dish toward which less juice was spreading. Eight of the ten individuals came near to the bag and extended their proboscides, the latter reaction always occurring before the siphon touched the cheese cloth. No behavior of this sort was observed at the opposite end of the dish, although six individuals came into contact with the unbaited packet, touching it ten times during the hour. They always moved away from it and no proboscis activity took place.

Other experiments of a similar kind, varying somewhat in details, were carried out, and gave essentially the same results. That dilute materials emanating from fish meat stimulate Alectrion and cause the animal to thrust its proboscis, in short, that the snail scents distant food, was clearly demonstrated. Whether the final discovery of the food is the result of chance movements, or of some directive influence of olfaction, is a problem which will be discussed later, particularly in connection with the reactions of *Busycon*.

The responses of Alectrion to distant food in a current of water were next investigated. Dimon ('05) describes the activities of the mud snail when in a stream containing food juices which flowed down a beach, and concludes that "if a current flows from the food to a snail, the animal will crawl up toward the food." The same author also finds, however, that the snail "shows a tendency either to move against a moderately strong current, or to orient itself when at rest with its head pointing against the current." The reader, therefore, is left somewhat in doubt as to the significance of the snail's movements against the current in the first instance, and may ask: Were they affected by the presence of food up the stream?

In order to determine whether food juices added to the water caused Alectrion to move more frequently against the current,

the following tests were made: A wooden box open at the top, nearly one hundred and seventy centimeters long and about twelve centimeters wide, was lined with glass, and placed in a horizontal position on a table. A rubber tube for conducting sea water was inserted in one end of the box near the bottom. The water flowed the length of the box and out an opening at the opposite end. The current was moderately strong, and care was taken to keep it as uniform as possible throughout the experiment.

A snail whose reactions were to be studied was placed in a position near the middle of the box, and all its movements with or against the current recorded until it passed a line seventy-five centimeters, either down or upstream, from the starting point. Each animal was first tested four times in the current without food. Two, which moved against the current to the seventy-five centimeter line in all their trials, were discarded, as the experiment necessitated the selection of individuals which showed at least some tendency to move in the direction of the current. Five animals were finally selected which answered the requirements. After determining their responses to the current alone, they were tested again with two fish (*Fundulus*) placed near the head of the stream above the seventy-five centimeter line. The fish were constantly bathed by the water after it left the tube, but were so situated on the right and left margins of the stream as not to interfere with the force of the current. They were cut open, and were turned over or moved from time to time during the tests in order to disperse the juices more freely. In trial one, throughout the experiment, the snail was placed heading across the current, so that its initial impact was on the animal's left side. It was started in the opposite direction in trial two; in trial three it was headed down stream and in trial four, upstream. The position of the snail at the beginning of the test, however, seemed in no way to affect its later activity and the final result. A single snail was given but one trial a day, and all were kept in good physiological condition by occasional feeding.

Table 1 shows the result of the experiment.

TABLE 1

Showing the distances in centimeters moved by mud snails and their arrivals up and down stream, when started midway between two lines 150 cm. apart in a current with and without food juices

| ANIMAL NUMBER | TRIAL NUMBER | IN CURRENT WITHOUT FOOD JUICES | | | | IN CURRENT WITH FOOD JUICES | | | |
|------------------|-----------------|--------------------------------|----------------|--------------|----------------|-----------------------------|----------------|--------------|----------------|
| | | Distances moved | | Arrivals | | Distances moved | | Arrivals | |
| | | Up stream | Down stream | Up stream | Down stream | Up stream | Down stream | Up stream | Down stream |
| 1 | 1 | 75 | 0 | + | | 80 | 5 | + | |
| | 2 | 0 | 75 | | + | 75 | 0 | + | |
| | 3 | 0 | 75 | | + | 75 | 0 | + | |
| | 4 | 0 | 75 | | + | 81 | 6 | + | |
| 2 | 1 | 0 | 75 | | + | 75 | 0 | + | |
| | 2 | 90 | 15 | + | | 117 | 42 | + | |
| | 3 | 120 | 45 | + | | 119 | 44 | + | |
| | 4 | 51 | 126 | | + | 134 | 59 | + | |
| 3 | 1 | 18 | 93 | | + | 75 | 0 | + | |
| | 2 | 116 | 41 | + | | 112 | 37 | + | |
| | 3 | 0 | 75 | | + | 63 | 138 | | + |
| | 4 | 7 | 82 | | + | 173 | 98 | + | |
| 4 | 1 | 0 | 75 | | + | 61 | 136 | | + |
| | 2 | 75 | 0 | + | | 4 | 79 | | + |
| | 3 | 0 | 75 | | + | 80 | 5 | + | |
| | 4 | 15 | 90 | | + | 10 | 85 | | + |
| 5 | 1 | 53 ¹ | 0 | — | | 77 | 2 | + | |
| | 2 | 63 ² | 0 | — | | 75 | 0 | + | |
| | 3 | 87 | 12 | + | | 75 | 0 | + | |
| | 4 | 0 | 75 | | + | 75 | 0 | + | |
| Total | | 770 | 1104 | 6+ | 12 | 1636 | 736 | 16 | 4 |

¹ Closed trial with snail resting at 53 cm. line.

² Closed trial with snail resting at 63 cm. line after starting it once by touching it.

Of the twenty preliminary trials in the current alone, there were eight in which the snails either arrived at the seventy-five centimeter line upstream, or showed a marked tendency to move against the current. The number of arrivals upstream was doubled, however, when the fish were placed near the head of the current. The five snails traveled 770 centimeters against

the current without food and 1636 centimeters with the food present. The distances moved across the current are not recorded.

A snail failed in but one case to increase its arrivals upstream when the fish were there (Animal number four). It should be noted, however, that this individual showed a greater tendency to move against the current when food was present than it did in its absence. The force of the current infrequently caused a snail to lose its foothold and slip a short distance on the glass, but it soon recovered itself and continued its locomotion. The total distance moved as a result of slipping in the forty trials was but little over fifty centimeters, over forty of which were recorded for this same animal in its trials with food juices in the water.

The average time taken by the snails in reaching the seventy-five centimeter line up the current without food, in six trials, was approximately twenty-seven minutes, and with food present, in sixteen trials, twenty-nine minutes.

The experiment indicates that snails which are inconstant in their reactions to a current, or more often go downstream, move more frequently against a current when it carries dilute food juices; in truth, they exhibit olfactory responses leading to the discovery of distant food. When the animals tested were allowed to continue their progress against the current beyond the seventy-five centimeter line, they usually arrived at the fish and began feeding.

2. Organs sensitive to food extracts

Having made certain that *Alectrion* responds actively to dilute food stimuli, experiments were begun to determine the sensitiveness of the external parts of the snail to odorous material, in the hope of discovering the olfactory receptor.

An extract of fish meat was prepared by grinding muscle tissue of *Fundulus* in sea water and filtering the product. A small amount of dry carmine was added to the filtrate, in order to make it visible in water.

The tentacles. The tentacles were first tested. When the fish extract was squirted over the tentacle of a moving snail by

means of a finely drawn out pipette, a marked reaction followed. The tip of the tentacle coiled rather violently, the animal stopped, the siphon was swung into the stimulating material and the proboscis extended and worked over the bottom of the glass dish in which the tests were made. Numerous animals were tested, and although considerable individual variation in respect to sensitiveness to the stimulus was noted, the reactions were remarkably constant, as the following responses of five snails will show. Sea water mixed with carmine was always applied with a special pipette to the tentacle before the fish juice in order to make sure that the reactions observed were due to chemical rather than tactile stimulation. The snail was moving when the test was made, and at least a minute elapsed between each trial with the fish juice. The material was applied five times to the right tentacle, and then five times to the left one, and care was taken to have the snail in water free from the stimulating substance when the test occurred. In fifty trials, ten with each individual, there were forty-seven reactions as described above. One failure to respond was noted, and two cases where the proboscis was extended but locomotion continued. The animals scented the juice, therefore, forty-nine times in fifty trials.

To sea water and carmine the snails usually responded, if at all, by twitching or coiling slightly the tips of their tentacles without cessation of locomotion. With the exception of one doubtful case, the proboscis was protruded but three times in the fifty trials, and then without the animal stopping as it characteristically did when stimulated with fish juice.

Another method for comparing the effect of a pure tactile stimulus with that of one accompanied by a chemical stimulus was as follows: A small piece of cotton was rolled into a ball and placed in the open end of a pipette. Some filtered *Fundulus* extract was then put into the pipette back of the cotton. By exerting slight pressure on the bulb it was possible to flood the cotton with juice, which then could be applied locally to any part of the snail's body. A pipette plugged with cotton and holding sea water was used in a similar way when a pure tactile stimulus was required.

The tentacles of a slowly moving snail were touched ten times with each of these pipettes, the trial with the fish extract always following that with sea water. The stimulating agent was applied to the right and left tentacle alternately. In four instances the cotton flooded with sea water excited no response; once the proboscis was momentarily extruded; and in the remaining trials the snail usually turned slightly toward the cotton, sometimes bending the siphon in its direction, and continued its course. Some slight coiling of the ends of the tentacles was also observed, and once the snail stopped moving after stimulation.

When the cotton flooded with fish juice was applied to the tentacles, the reaction was quite different and similar to that obtained by squirting the fluid on these organs. They contracted and coiled, the siphon was bent toward the cotton and the animal turned in the same direction, often following it the greater part of a circle. Finally, the proboscis was extended in the direction of the juice. The proboscis reaction occurred in every trial.

The foot. The sensitiveness of the lateral and posterior borders of the foot was tested by the same methods. A stream of sea water containing dry carmine, when applied as gently as possible to the side of the foot of a moving animal, caused either no visible response or a slight local contraction at or behind the spot first stimulated. Sometimes the contraction wave passed posteriorly, causing a lifting or withdrawal of the edge of the foot. Fish extract and carmine, on the other hand, induced a well marked local contraction, often resulting in a lifting of the border of the foot, the raised area extending backward until the posterior end of the foot was free from the glass. Once or twice the contraction wave ran forward, causing a lifting of the antero-lateral border of the foot.

When the extract was applied to the posterior end of the foot of a moving snail, the response was also well marked. The end of the foot for a distance of two or three millimeters, or less, was lifted from the glass and perhaps slightly contracted. This reaction very seldom occurred when sea water and carmine were used.

When the foot was stimulated by the use of a pipette plugged with cotton, little or no difference in the reaction to the cotton flooded with fish juice and to that with sea water could be discerned. On stimulating, for example, the posterior tip of the foot, it was drawn forward under both conditions, the extent of the contraction evidently depending largely on the force of the contact.

In all these tests upon the snail's foot, one fact was very striking; the animal failed to extend its proboscis in search of food, as it characteristically did after fish extract was applied to the tentacles. The next problem, therefore, was to determine whether stimulation of the tentacles was necessary in order to call forth the proboscis reaction. Accordingly the tentacle bases of several snails were cut off as near the head as possible, and in all cases, proximal to the eyes. Removing the tentacles did not modify in any recognizable way the normal behavior of the animals, which, therefore, could be experimented upon shortly after the operations. In the absence of the tentacles, the fish juice and carmine were applied with a pipette to the lateral extensions of the anterior border of the foot already described. In fifty tests, twenty upon one animal and ten each upon three others, the proboscis reaction took place forty-two times. Only two such responses to sea water and carmine occurred in fifty tests.

The fish extract brought forth a very characteristic series of movements, which occurred time and time again. The lateral process of the foot contracted after stimulation, the moving animal then stopped, depressed its siphon into the juice and finally extended its proboscis. Often there was no response to the sea water and carmine and usually, at the most, but a slight contraction of the foot process as the animal continued locomotion. The differences in behavior were pronounced. That the proboscis reaction was not dependent on chemical stimulation of the tentacles was clearly shown by these and many other tests, some of which will be described.

The siphon. The movement of the siphon into the fish extract was one of the most striking features of the snail's behavior,

both with and without its tentacles, and it soon became evident that the animal was in some way testing the stimulating material by means of this organ. By using great care in the application of the stimulating fluid to the foot processes of snails deprived of their tentacles, the proboscis reaction was seen to occur when the siphon clearly entered the fish juice, whereas when the siphon was not depressed or swung far enough to reach the extract, the reaction did not take place. In one instance, for example, the extract was twice applied to the foot when the siphon was well raised in the water. Each time the foot process contracted, and the siphon was swept downward in the direction of the extract; but not reaching it, there was no protrusion of the proboscis. On the third trial the siphon was depressed farther, entered the juice and the proboscis reaction followed. Although sea water and carmine sometimes caused a contraction of the foot process, there was no well marked downward movement of the siphon such as occurred in the tests with fish juice.

The dorsal surface of the siphon was next tested. When touched with cotton, placed in the end of a pipette and flooded with fish extract, the siphon was bent backward and upward toward the cotton. This movement was often accompanied by a lifting of the tentacles and anterior end of the foot, as if the snail were stretching upward to reach the stimulating material. To a pure tactile stimulus the snail usually responded by contracting the siphon somewhat, but much less tendency was shown to bend it upward. In ten trials the proboscis was extended but twice, and in one of these cases at least an excess of fluid was applied. Another individual did not respond to stimulation of the dorsal surface of the siphon, but showed ten proboscis reactions in the same number of trials after stimulation of the ventral surface of the organ by the same method. When, however, cotton flooded with sea water was applied to this region, either no response was noted or there was a tendency shown to draw the organ away from the cotton. Since the ventral wall of the siphon is split its entire length, it was impossible to determine by this procedure whether the proboscis thrust was evoked by stimulation of the under part of the siphon, or of some

receptor within the mantle chamber to which the fluid was conducted.

The results of the experiments reported above indicate that the skin of *Alectrion* is in general more or less sensitive to food materials, and that certain regions of it, such as those covering the tentacles, the siphon, and lateral foot processes, sometimes at least play a part in reactions to food, inasmuch as stimulation of these surfaces by its juices initiates a testing of the environment by means of the siphon. Finally, appropriate stimulation of the under surface of the latter organ, or of one within the mantle chamber, induces a protrusion of the proboscis. To further test these conclusions, and to localize more definitely the receptor associated with the siphon or mantle, it became necessary to limit in some way the action of the siphon. This end was accomplished by tying and cutting off the organ.

The following tests were made upon a snail with its tentacles removed, and a thread tied around the siphon, so that about one and a half millimeters of the organ were visible between the anterior margin of the shell and the thread. It was noted that the slit-like aperture on the under side of the siphon posterior to the thread opened and closed, indicating that water could still pass into the mantle chamber through the proximal portion of the organ. Less than half an hour after tying the siphon the snail was moving about with the organ extended in normal fashion. Fish extract and carmine applied to the lateral foot processes induced the proboscis reaction in five trials out of six. The proximal part of the siphon was bent down into the juice after the contraction of the foot process, and before the extension of the proboscis. In one trial the siphon was only slightly moved, and no further response ensued.

A second thread was then tied around the siphon about one millimeter back of the first. Several tests made shortly after this operation, and on the following day, gave similar results. The proboscis was extended when the base of the siphon was definitely moved into the fish extract. In an attempt to tie a third thread about the siphon, the organ was pinched off just back of the second thread. It was now slightly over one milli-

meter in length, and open at the end as well as below. Five stimulations of the foot processes failed to produce the proboscis reaction. The processes contracted as before, but the stimulus was not adequately tested with the stub of the siphon. When, however, a piece of fish muscle was placed in front of the snail, and when the juice was applied beneath the siphon, the proboscis reaction occurred. Repeated stimulations of the foot processes resulted in a limited number of proboscis thrusts, and on the following day, the animal's responses were again studied very carefully. In twelve trials there was only one typical proboscis reaction following the stimulation and contraction of the foot processes, and in this case the juice was squirted too far forward beneath the siphon. In three trials the proboscis was momentarily extended as the snail moved away from the extract, as if it had been only weakly stimulated, and in one trial the animal turned slightly toward the stimulating material when the proboscis was thrust once. In the remaining trials, although the foot processes contracted, the extract was not tested by the siphon stub, and the proboscis did not appear. When, however, after these tests and on the following day, the extract was dropped in front of or into what was left of the siphon, the proboscis reaction followed. Ten tests were made. This snail was again experimented upon in the condition described above, and also with a thread tied around the end of the cut siphon, which of course restricted its action still more. It should be understood that, on account of the slit in the ventral wall of the organ, it was impossible to render the extreme proximal part completely functionless. Whenever the stimulation of the foot process caused the animal to stop and test the environment with the base of its siphon, the characteristic proboscis protusion took place. Every trial with fish extract was preceded by one with sea water and carmine, but the latter never called forth the proboscis reaction.

Several other individuals, with the siphons for the greater part of their length tied or cut off, and with and without tentacles, were carefully tested in a similar way, but as the results obtained were in accordance with those described, it is unnecessary to

report them in detail. In the case of those snails which possessed their tentacles, but had their siphons shortened, the proboscis reactions were of greater frequency, as the tentacles are particularly sensitive, and their stimulation was usually followed by a more thorough investigation of the surroundings with the siphons.

That dilute food juices taken into the siphon or mantle cavity cause *Alectrion* to respond actively was demonstrated in another way. When a snail is placed in an aquarium, the bottom of which is covered with sand, it usually soon buries itself completely except for the tip of the siphon which projects slightly above the surface and conducts water to the gill within the mantle chamber below. If fish extract and carmine are squirted over the end of the siphon, they disappear within, and the snail often reacts by coming to the surface and extending its proboscis. Different individuals were found to vary considerably in their reactions, certain ones always responding to the stimulus in a marked way, whereas others were more erratic in their behavior. In one instance, in over sixty trials involving many individuals, a snail came out of the sand immediately after sea water and carmine were squirted over the siphon tip, but in this case the proboscis was not extended as it was when the animal responded to fish juice. Snails deprived of their tentacles showed the same behavior as others; in fact, it is unlikely that the stimulating material reached any anterior portions of the body outside of the mantle chamber and siphon when the animal was buried in the sand.

Conclusions. The experiments upon *Alectrion* all substantiate the conclusion already drawn, namely: that the reaction to food juices, consisting of an extension of the proboscis, is under the conditions of the tests the result of the stimulation of a receptor located either at the extreme base of the siphon or within the mantle cavity. The skin receptors of the foot, siphon and especially the tentacles are affected by these materials has been clearly demonstrated, but the part they play in the final reaction appears to be subordinate to some organ which, judging from the animal's response, is much more sensitive to stimulation by dilute food substances. As a result of skin sensitiveness, a change

in the chemical surroundings of a snail may sometimes lead to a testing of the environment by this olfactory receptor. That the organ sought was the osphradium, which is located on the mantle at the base of the siphon, appeared highly probable, but the small size of *Alectrion* made an experimental study of the function of this organ impracticable.

Certain peculiarities of the reactions recorded above will be more fully explained in the discussion of the senses of taste and smell.

BUSYCON CANALICULATUM

In order to investigate further the part played by the siphon system in olfaction with special reference to the function of the osphradium, and secondly to determine how a snail finds distant food, the study of a second species was undertaken. For these purposes a whelk, *Busycon canaliculatum* (Linn.), better known by the generic name *Sycotypus*, was selected, principally on account of its large size, but also for its well marked reactions to food juices and its general structural resemblance to *Alectrion*. The most conspicuous external morphological difference between *Alectrion* and *Busycon* is the form of the shell, which in the latter species is drawn out anteriorly like an inverted gutter, covering dorsally all but the tip of the pallial siphon. This shell siphon protects and at the same time restricts the movement of the pallial siphon.

1. Organs sensitive to food extracts

When the study of *Busycon* was first begun, I was unaware that the oyster often formed a conspicuous part of its natural diet, and the discovery that oyster extract called forth a marked response was quite accidental. Food juices procured by grinding fish meat in sea water, when squirted in front of a snail, induced a reaction involving the protrusion of the proboscis. Accordingly, the first tests were made with this stimulus. During the course of the experiments it happened that some oysters were available, and the effect of oyster juice was tried. The resulting reaction was so sudden and pronounced that henceforth only

oysters and oyster juice were used in feeding and stimulating the snails. The extract was prepared by first removing the soft parts of the oyster from the shell, and placing them in a small amount of sea water. This was usually done the day before the material was used. The oyster was then ground in the sea water with a pestle and the extract filtered out.

The foot, mantle and tentacles. All of the external soft parts of the body of *Busycon* which were tested were found to be sensitive to food juices. The snails frequently crawled up the sides of the aquarium and rested at the surface partly out of the water. In that position the anterior margin, and sometimes a portion of the under surface of the foot could be readily tested. Dropping sea water on these exposed parts of the foot produced little or no effect, whereas a single drop of oyster extract called forth a well marked local contraction of the stimulated area.

By removing the snails from the water, other portions of the body surface were rendered more accessible. The animals were placed on their backs and sea water dropped from a pipette around the mantle edge and on the proximal external surface, and the partly opened distal end of the siphon. There was no reaction noted except at the siphon tip, where there sometimes occurred a very slight contraction. Drops of oyster juice, however, caused contraction of the mantle edge and the surface of the siphon. When the end of the siphon was stimulated, it was drawn back farther into the shell. The last was the most marked reaction.

As in *Alectrion*, the tentacles of the whelk were found to be very sensitive to food extracts. When stimulated under water with fish or oyster juice, the tips contracted and usually were bent somewhat to one side. When fish extracts mixed with carmine was squirted over a tentacle, or the anterior margin of the foot of a snail resting in the water, the proboscis was usually extended, after a time, near the stimulated region. The same reaction occurred after the tentacles had been removed when the juice was applied to the anterior part of the foot and the regions beneath the base of the siphon. A notch in the foot margin was formed by the local contraction and lifting of the stimu-

lated area, and the proboscis was thrust downward through the opening.

In these tests it was impossible to determine definitely whether the extract entered the base of the siphon before the proboscis was extended, but since the reaction was characteristically slow and a diffusion of the stimulating material took place, it seemed likely that it did. Moreover, when a snail was on the glass side of an aquarium, where the ventral surface of the siphon could be observed, it was noted that the lips of the organ spread apart near its base when it entered oyster extract which was being applied to the tentacles or foot; in fact, the split condition of the siphon appears to be partly an adaptation for taking in materials which are close to the body. The part played by the chemical receptors of the tentacles and foot in responses to food stimuli will be fully considered in the succeeding account of the snails' behavior.

The siphon system. The most striking reaction of all was obtained by squirting the oyster extract in front of the end of the siphon so that it was taken into the organ in considerable amount. In the case of a moving snail, stimulation in this manner almost always caused it to increase its rate of locomotion, and to swing the siphon farther to the right and left. The lateral swinging of the siphon is characteristic of the moving animal and will be described in more detail later. Continued stimulations were usually sooner or later followed by an extension of the proboscis.

When the extract was applied in the same way to a resting animal, in most instances it caused locomotion and the proboscis reaction. Even when the snail was much contracted the same response was noted. In all cases the animal was first tested with sea water squirted from a pipette, which invariably failed to produce the reactions described above. Stimulation of a resting snail as large as *Busycon* by the pipette method was particularly satisfactory, as the stream of oyster juice could usually be directed over the end of the siphon without coming in contact with the tentacles or other parts.

Busycon, like the mud snail, has the habit of burying itself in the sand. When placed in an aquarium provided with a sandy bottom, it may enter it immediately, or in other cases after a time. A portion of the shell may remain exposed, but more often the snail goes deep into the sand so that only the tip of the pallial siphon is visible above the surface.

The response of a buried *Busycon* to food particles has been described by Colton ('08) as follows:

The *Sycotypus* had not been fed for a month or so and was buried in the gravel. To stimulate, I added some very finely chopped-up oyster to the aquarium. When it started to crawl out of the gravel, a few minutes after I added the oyster juice, I placed some live oysters in the aquarium with it. It attacked one of the oysters five minutes after I placed them with it.

Buried snails were stimulated many times by squirting oyster extract over the exposed siphon tip, and often the animal responded by emerging completely from the sand after the manner of the mud snail. Sea water applied in the same way usually had no effect whatsoever, the most marked response noted being a contraction of the end of the siphon. In no instance did the snail come out of the sand. The usual reaction to oyster juice was first a flaring and stretching upward of the end of the pallial siphon, followed by a lifting of the shell siphon and the final emergence of the rest of the body. Protrusion of the proboscis often occurred.

The following test illustrates the reaction well, although it was a slow one: The snail was buried so that only the top of the shell and the siphon tip were visible. The latter was in contact with the glass side of the aquarium. Oyster extract squirted over the end of the siphon after a time caused, first this organ and the shell covering it to be lifted, and later the snail to come out of the sand. It then began to crawl up the side of the aquarium, where the juice had been applied, with its proboscis slightly extended. It was thrust out farther after more extract was taken into the siphon, and upon stopping the application of the substance, the snail ceased moving and worked its proboscis over the glass. It could not be made to crawl farther

upward by squirting sea water over the siphon, but more oyster juice taken into the organ caused immediate locomotion to the top of the aquarium, where it was fed.

In many instances the response of the buried snail was almost startling in its suddenness, the animal pushing its way upward through the sand with its powerful muscles, and appearing entirely free on the surface within a minute or two after stimulation.

2. The finding of distant food

That *Busycon* is remarkably successful in finding food is only too well known to the lobsterman, who frequently finds his traps containing more whelks than lobsters. One fisherman whom I interviewed told me that he had taken as many as twenty in one lobster pot in a night, and that oily bait such as mackerel, swordfish and bluefish attracted them in the greatest numbers. At the time, he was sorting over hundreds of specimens contained in two large cars in view of shipping them away to be cut up and used for fish bait. The entire collection had been gathered from his lobster traps.

Sumner, Osburn and Cole ('13) record the capture of "fifty-one large specimens" in "three lobster pots in a single day."

How *Busycon* finds distant food is, therefore, a problem of peculiar interest and one which was carefully investigated experimentally. Before attempting to answer this question, two experiments, demonstrating the ability of the animal to find food in an aquarium, may be briefly described.

A snail was placed in a circular glass jar measuring approximately twenty-seven centimeters in diameter and twenty-five centimeters in height. A ball of cheese cloth was hung over the rim of the jar so that it dangled in the water near the surface. Opposite this was suspended in a similar way a cheese cloth bag containing the soft parts of an oyster, which was slightly squeezed to facilitate the diffusion of juices. The snail moved slowly around the periphery of the bottom of the jar. Passing beneath the baited bag, it turned back stretching its siphon upward. It then circled about showing the increased activity characteristic

of scenting behavior. Presently the proboscis was extended and the anterior end of the foot raised, as if it were attempting to climb up to the bag. The lifting of the siphon was also very marked. After moving away and returning two or three times, it finally succeeded in gaining a foothold on the side of the jar directly beneath the bag containing the oyster, and crawled upward in a straight line until the siphon tip touched the bag. It was then offered a piece of oyster which it speedily devoured. No attention was paid to the unbaited ball of cheese cloth.

Other experiments were carried on in a larger square wooden tank which was about seventy-four centimeters wide and slightly over eighteen centimeters deep. Sea water entered through a rubber tube suspended near one side of the tank. The end of the tube rested on the bottom, and was so arranged that the current first swept across a portion of the tank before eddying about in various directions. The water overflowed at the top of the tank.

Three snails were placed in the aquarium, and when the test was made, all were resting. Then a piece of cheese cloth containing an oyster removed from its shell, was tied over the end of the tube so that the water flowed over the oyster and through the cloth. Within a minute and a half, all three animals were in motion. Two minutes and a half later two of the snails touched the cheese cloth with their siphons. One of them was removed, and the third snail arrived at the tube only a minute and a half afterward. Thus all the animals located the source of the odor in less than six minutes. As they moved actively about the aquarium their tentacles curved downward, and their siphons swept alternately to the right and left.

Other snails, which were tested in the same way, showed similar responses. After the water had been running for about a half hour and the juices of the oyster had been considerably dispersed, they exhibited no tendency to move toward the source of the current.

These tests indicate that *Busycon* finds food not by accidentally coming upon it as a result of random movements, perhaps initiated by olfactory stimulation, but, on the contrary that

it is in some way directed toward it. From the experiments reported in the preceding pages, it is equally clear that the receptor particularly concerned with reactions to dilute chemical materials is unquestionably situated within the siphon, or its proximal continuation, the mantle cavity, a conclusion which was finally confirmed. With these facts in mind, the problem of how the snail is directed toward distant food can now be approached.

Attention has already been called to a conspicuous habit of *Busycon* associated with locomotion, that is: a lateral swinging of the siphon to the right and left. Not only the siphon, but the entire body above the upward neck-like extension of the foot, is involved in this movement. The foot proper is the only portion unaffected. The motion may be likened to the swinging of a compass needle before it comes to rest, the pivot, in the case of the snail, being the dorsal neck of the foot supporting the shell and associated organs, the needle point being represented by the siphon tip.

It soon became evident that there was a marked difference in the direction taken by a moving animal dependent on whether stimulation by food juices occurred when the siphon was at the end of its swing to the right or to the left. When oyster extract was liberated in front of the siphon, when swung farthest to the right, the anterior end of the foot turned in the same direction and the animal, therefore, circled to the right. To cause the snail to turn in the opposite direction, it was only necessary to apply the stimulating material when the siphon was swung to the left. When, however, the juice was squirted in front of the animal: that is, in a position so that the moving siphon end passed through it in the middle of its way from one side to the other, the snail tended to follow a straight course ahead. By appropriate stimulations, therefore, it was found that a snail could be actually led about the aquarium in any direction, and even be made to leave the bottom and crawl up the side, provided the siphon first pointed in the direction which it was desired the animal should take. In my notes are recorded over one hundred and forty cases where snails were directed from the bottom to one

of the vertical sides of the aquarium, in which position they were often fed. These tests were made on over seventy individuals. An analysis of the snail's movements, when responding to olfactory stimuli, shows clearly that the swinging of the siphon precedes stimulation, and the latter precedes change in the direction of locomotion.

In the experiment already described, where the odor was distributed through the aquarium by a current of water flowing over an oyster held to the end of a tube, the same sequence of muscle activity and stimulation was noted. One of the snails, for example, was moving somewhat diagonally across the current when the siphon was swung to the right and held into the stream. The anterior end of the foot then turned to the right, and the snail circled in the same direction to the bag containing the oyster.

A study of the reactions which occurred when a snail was directed from the bottom to the side of the aquarium, and its responses in the latter position, were particularly instructive. Certain trials, therefore, may be described in some detail.

A snail was moving slowly over the floor of the aquarium. When oyster extract was liberated in front of the siphon by the use of a pipette, so that the end of the organ passed through the extract in the middle of its path from one side to the other, the animal took a straight course across the aquarium. The siphon was swung rather close to the bottom of the aquarium, so that its tip, bent downward, touched at the right and left terminations of its movements. Upon arriving at the side of the aquarium the left surface of the end of the siphon struck the glass first, and while it was in that position the stimulating material was applied. The animal tried to force the siphon to the left, but meeting an obstruction, raised it higher on the glass. Again the oyster juice was squirted over the siphon tip while in contact with the glass. The anterior end of the foot was next applied to the glass, and the snail began crawling upward. At the same time the siphon was swung far off the glass to the right. When it returned, it hit the glass still higher, as all the time the foot was moving upward. In the last position it was again stimulated, and the animal continued its upward course. The aper-

ture leading into the sac surrounding the proboscis was seen to flare, a reaction often noted when an animal was responding to an olfactory stimulus, and the proboscis was not fully extended. Upon arriving at the surface of the water, it was offered a piece of oyster which it ate. In cases where a snail was successfully directed from the bottom to the side of the aquarium, the procedure and reactions were much the same as in this instance.

In order to show, on the other hand, that a snail could not be directed upward until after the free end of the siphon was raised, another test may be described. After appropriate stimulations, the snail moved to the side of the aquarium, but then failed to lift its siphon. The oyster extract squirted over the siphon tip caused the animal to move parallel to the glass side, the right border of the foot being actually on it. Whenever the end of the siphon was slightly raised the stimulating material was applied, and finally the whole foot was fastened to the glass. In this position the siphon was moved very little, and stimulation resulted only in locomotion along the side of the aquarium close to and parallel with the bottom. The proboscis was extended, showing conclusively that the animal was scenting food juices. It eventually arrived at the end of the aquarium where the siphon was swung to the right. Upon applying the stimulus with the siphon pointing upward, the foot turned in the same direction and there was no further difficulty in leading the animal to the surface.

A third case illustrates how a snail can be made to circle while crawling on a vertical surface. The animal tested was resting by the side of the aquarium, the left surface of the end of the siphon in contact with the glass. Sea water squirted into the distal aperture of the siphon produced no reaction, but oyster juice caused locomotion. The latter was then liberated over the end of the organ when it was raised on the glass, and although it was swung off the side to the right once or twice, the foot turned sharply to the left, and the snail began crawling upward in the direction of the extract, as in the other instances recorded. In its upward course it moved slightly to the right of a vertical line, and the siphon was swung to the right and left in the usual

way. The oyster extract was next applied whenever the siphon was swung downward, that is, at the end of its dextral movement, and the foot responded by turning in the same direction. After the animal had definitely changed its course in this manner, the juice was squirted over the siphon tip when at the end of its swing to the left. The reaction was striking. The snail again headed upward, and on continuing the stimulation in the same way, circled to the left until the siphon actually pointed downward and the foot nearly so. In that position the foot slipped on the glass, and the animal slid to the bottom. By appropriate stimulation, therefore, a snail can be made to circle on a vertical surface as readily as on a horizontal one.

When *Busycon* was crawling on the glass side of the aquarium, it was comparatively easy to observe the details of its reactions. The turning of the anterior end of the foot was seen to occur very quickly after the application of the extract to the siphon tip at or near the end of its lateral movement. If, for example, stimulation took place when the siphon was swung to the left, the foot was turning in the same direction synchronously with the return of the siphon to the right. It appeared also as if the return swing of the siphon was somewhat shortened as a result of the preceding stimulation, or possibly the turning of the foot in the opposite direction.

Whether the eyes or chemical receptors of the tentacles or foot play a part in these responses may next be considered. The contention might be raised that some of the oyster extract passed back along the sides of the siphon and by stimulating the tentacles directed locomotion. To remove the tentacles, bearing the eyes at their bases, is a simple operation which, as in *Alectrion*, seems to have no injurious effect on the snail. Soon after the operation the animal will take food and its behavior appears quite normal. A snail, whose tentacles and eyes had been eliminated by severing the tentacle bases close to the head, was tested with oyster juice squirted from a pipette in front of the siphon. Its scenting reactions were identical with those of animals possessing tentacles. It was led about the aquarium and up the side by the same method used in directing the movements of other

snails. When resting, sea water squirted over the siphon tip induced no locomotor response, whereas oyster juice stimulated the animal to activity and induced the proboscis reaction. It was noted, however, that the snail sometimes had difficulty in securing a piece of oyster which was placed in front of it. Its behavior emphasized what had already been observed, viz: that the tentacles aid the foot in the sensing and manipulation of food before it is taken into the mouth.

It has been shown that the foot of *Busycon* is sensitive to food juices. Accordingly, tests were made to determine whether the direction of locomotion could be controlled by applying the oyster extract to this organ. A snail, which was resting in a corner of the aquarium, could not be stimulated to locomotion by squirting the extract on the foot margin or on one of the tentacles. Siphon stimulation, however, caused the animal to crawl up the end of the aquarium and to extend its proboscis. It was then led over to the side where it moved diagonally upward after stimulation. Oyster juice was now applied to the right antero-lateral margin of the foot, but without directive effect, for the snail continued in its original course. Another animal was moving in a similar direction up the side of the aquarium. This time, the juice was applied to the left anterior margin of the foot, but the animal turned to the right and downward, or in a direction away from the location of the stimulating substance. By squirting the juice in front of the siphon whenever it was swung to the left, the animal was made to turn upward, and was soon crawling in the direction taken at first. Stimulation of the siphon system and not the foot, therefore, was effective in directing the snail toward the food juices. In the course of these and other tests, it was found that the application of oyster extract to a limited portion of the anterior border of the foot of a moving snail often caused that part to slow down momentarily; a reaction which may be compared to the local contraction of the stimulated foot margin of a resting snail already described and one which, if continued under the influence of a more concentrated stimulus resulting from actual contact with food, would end in food procurement by the characteristic method of partly

surrounding it with the anterior end of the foot. Oyster juice placed in front of the siphon, however, always caused the retarded area to move forward again at normal speed.

The results of all the experiments, designed to show how dilute food materials direct the movements of *Busycon*, indicate that the receptor concerned in the reactions is associated with the siphon rather than the tentacles or foot. This conclusion is supported by other tests to be described.

When the pipette method of stimulation is used, it is conceivable that the stream of fluid directed over the siphon tip, no matter how carefully applied, may act as a tactile stimulus, and thus introduce a factor other than a chemical one, which may play a part in determining the response of the snail. That such a tactile stimulus alone is incapable of bringing about the reactions described is perfectly clear from the failure of the animals to respond to sea water, when substituted for oyster juice. In order to eliminate a possible tactile influence when fluid is forced from a pipette, the soft parts of an oyster were tied up in a piece of cheese cloth, and held by a thread over the end of the siphon. The two snails tested in this manner became very active and were led from the bottom of the aquarium up the side without difficulty. They followed the juice slowly diffusing from the bag as readily as when it was squirted from a pipette.

The last experiment suggested a similar but more satisfactory method for studying the directive influence of olfaction in *Busycon*, and one which led to some of the most striking results of the investigation. Two small sticks, of equal length, were fastened to the distal end of the shell siphon with dental wax in such a way that they projected beyond the shell and the tip of the pallial siphon when fully extended. One stick was directed approximately forty-five degrees to the right of the principal axis of the siphon, and the other forty-five degrees to the left. A piece of oyster was then tied up in cheese cloth, and fastened to the free end of one of the sticks three or four centimeters from the tip of the shell siphon, whereas a ball of cheese cloth, of the same size as the oyster packet, was tied to the end of the

other stick. Accordingly, when the apparatus was in position, and the snail moving, the baited bag was always in advance and lateral to the end of the siphon, and was balanced by the cheese cloth ball on the other side. When the siphon was swung to the right and left the packets were carried with it, and any disturbance in the water produced by their movement must have been the same on both sides of the siphon. From one packet, however, there occurred a continuous emanation of odorous material from the contained oyster meat, and although it must have been more or less diffused over the greater part of the arc described by the moving siphon, the most concentrated stimulating substance was continually ahead and to one side of the end of the siphon.

Accordingly if, as it appears, *Busycon* finds distant food by proceeding in the direction in which the siphon points when it receives the scent or the strongest scent emanating from it, the animal would be expected to circle to the right when the baited bag was on the stick directed to the right, and vice versa.

Table 2, indicating the results of eleven trials involving six individuals, shows conclusively that the snails circle in the direction of the bag containing the oyster. Only in one instance

TABLE 2

Showing the direction in which snails turned when cheese cloth containing oyster meat was tied to the end of a stick fastened to the shell siphon so that the packet was beyond and on the right or left of the tip of the pallial siphon

| TRIAL NO. | DATE | ANIMAL NUMBER | POSITION OF THE PACKET WITH OYSTER MEAT | DIRECTION TURNED |
|-----------------|-----------|---------------|---|------------------|
| 1 | August 3 | 1 | On the left | To the left |
| 2 | August 3 | 1 | Right | Right |
| 3 | August 3 | 2 | Right | Right |
| 4 | August 4 | 1 | Left | Right |
| 5 | August 5 | 2 | Left | Left |
| 6 | August 6 | 3 | Right | Right |
| 7 | August 7 | 4 | Right | Right |
| 8 | August 10 | 5 | Right | Right |
| 9 | August 10 | 5 | Left | Left |
| 10 ¹ | August 11 | 5 | Left | Left |
| 11 | August 18 | 6 | Right | Right |

¹ The snail was without tentacles in this trial.

(trial four) was there a failure in this response, and in that case the snail started to turn to the left before circling to the right, and behaved in a sluggish manner. In trial one the snail turned to the right after circling one hundred and eighty degrees to the left. In most instances the animals hit the side of the aquarium after turning about ninety degrees, but in trials one, nine and ten they circled one hundred and eighty degrees or over without meeting any obstruction. There seemed to be some tendency shown to turn more sharply when circling to the left.

Trial nine was so remarkable that it deserves special description. The bag containing oyster meat was tied to the left stick, and the snail immediately started forward turning to the left and rendering evidence that food was scented by exhibiting vigorous sweeps of its siphon. The tentacles curved downward, their tips now and then touching the bottom of the aquarium. Continuing to the left, the animal completed the first circle in four minutes without hitting the sides of the aquarium. After moving three-quarters of a second circle, it encountered the side, but swung clear and continued its course to the left, finally completing two and a half circles in eight minutes. It then came in contact with the aquarium side and lifted its siphon, thus bringing the packets above the end of the organ. Still following the scent, the snail crawled up the side of the aquarium, eighteen centimeters high, until both packets were actually lifted above the surface of the water. In this position the animal was fed with a piece of oyster.

A number of tests were made in order to determine whether the behavior of the snails just described could be interpreted in any other way than as responses to chemical stimulations. Animal number five (without tentacles) was observed while carrying sticks supporting two cheese cloth packets, neither one of which contained oyster meat. After several trials it became evident that the presence of the apparatus on the shell siphon did not produce any unusual behavior. There were no marked circling movements shown, and when slight deviation from a straight course took place it was as often in one direction as the other. When the animal, still carrying the packets, was stimu-

lated to activity with oyster extract, which was then swept away with a current of sea water flowing from the end of a rubber tube, the swinging of the siphon was more pronounced, and there was more tendency shown to move out of a straight course.

Finally, it was conceivable that some difference in the weight of the cheese cloth packet and the one containing oyster meat was a controlling factor in the responses described. Accordingly, samples of both types were weighed after taking up sea water, and it was found that the one composed entirely of cheese cloth was about one hundred and fifty milligrams heavier than the other. Tests were then made as follows: A pebble, weighing nearly three grams, was tied up in cheese cloth and fastened to the end of one of the sticks projecting from the shell siphon, whereas the other stick was left free. The difference in weight of the two was now nearly twenty times as great as in the original trials, yet the snail tested moved in a straight course irrespective of the side on which the pebble was suspended. The same apparatus was fastened to a second individual, the pebble being on the left stick. The snail crawled in an approximately straight line for about forty centimeters, when it was returned to the starting point. Oyster juice was then squirted in front of the siphon when it was moved to the left, and the snail responded by turning a half circle in the same direction. Again it was placed in the middle of the tank, and this time stimulated when the siphon was swung to the dextral side, after which it moved a half circle to the right. The snail made long sweeps with its siphon and was affected in no way discernible by the attached weight. These control tests show conclusively that the circling reactions of the snails described above were wholly due to the directive influence of olfactory stimulation.

The results of the experiments on *Busycon* support the conclusion that the animal, when moving in response to olfactory stimulation, is not directed toward food by juices falling upon external body surfaces such as those of the tentacles and foot, but that it takes a course in the direction indicated by the siphon when it receives the effective stimulating substance from a distant source. Assuming this conclusion to be correct, it is clear

that if the siphon were fastened to one side, so that it could not be swung over to the opposite side, the snail should move in a circle when reacting to food juices generally distributed in front of it. Accordingly, the following experiment was tried: One end of a string was fastened to the tip of the shell siphon, and the other end tied around the operculum, which lies behind the shell on the dorsal surface of the foot when the latter is expanded for locomotion. The string was adjusted so that the end of the siphon was drawn forty-five degrees to the left of its median position. It could be swung still farther to the left but not to the right. The right tentacle pointed directly forward, the left one in the same direction as the siphon. Contrary to expectation, restricting the movement of the siphon in this way did not seriously disturb the animal or cause it to circle in any abnormal manner. The test was begun by squirting oyster juice from a pipette over the right tentacle in front of the foot. The snail moved forward slowly a short distance, scented the juice and extended its proboscis. The extract was then placed alternately in front of the siphon tip, on the right tentacle and on the right anterior border of the foot. By this procedure the stimulating material was about equally distributed in front of the animal and on both sides. The snail began immediately to turn to the left, and soon completed a circle of about twenty to twenty-five centimeters in diameter. There was very little tendency shown to turn to the right, whereas siphon stimulation always brought about active locomotion to the left. The proboscis was extended somewhat during the test, indicating conclusively that the animal was sensing food and was not disturbed by the presence of the string limiting siphon movement. Stimulation was finally discontinued, and the snail promptly moved forward in a straight line for about twenty-five centimeters. Then for the first time it showed disturbance over the tied siphon, and began stretching upward and tugging on the string. As a result of this activity, it turned ninety degrees to the right and stopped moving. In the absence of the oyster extract, therefore, the only decided deviation from a straight course was one to the right. When given a piece of

oyster, the snail began feeding and gave no further sign of agitation. Another animal, whose siphon was tied as in the preceding case, responded to oyster juice placed in front of the end of the siphon by circling to the left. Owing to the curvature of the shell all attempts to fasten the siphon to the right of its median position were unsuccessful.

Before any further discussion of these results is undertaken, it seems desirable to give an account of an investigation demonstrating at least one function of the osphradium.

3. *The osphradium*

The study of the responses of *Alectrion* and *Busycon* reported in the preceding pages led to the belief that an olfactory receptor was situated somewhere within the mantle cavity, and accordingly an investigation of the part played by the osphradium in the scenting reactions of *Busycon* was finally undertaken.

Although the osphradium on account of its peculiar structure was formerly called a 'false gill,' it more recently has been termed an olfactory organ, or one for testing the quality of the water entering the mantle chamber. The evidence in support of the latter designation appears to be principally of a morphological character. In the first place, it was found that the organ was richly supplied with nerve endings and was undoubtedly a receptor of some sort, and secondly, its position on the mantle at the base of the pallial siphon would necessitate water taken in through the siphon coming in contact with it before reaching the gill. In this connection a statement by Dakin ('12) in his work on the morphology of *Buccinum* is of interest, since this genus possesses the same kind of osphradium in the same relative position as that found in *Busycon*.

One would imagine from observation of the living animal that the siphon was connected with some important system of sense organs. It is continually in motion from side to side, and extends much further from the animal and is more active than the tentacles.

Examination of the mantle cavity in this light reveals an interesting series of organs. The osphradium, which is a darkly pigmented structure on the left side, is situated right across the end of the siphon (fig. 8, *Osph.*). Thus all water entering the pallial cavity must pass over it *before reaching the other organs*.

The osphradium of *Busycon* is an elongated organ slightly over three centimeters in length, broadest in the middle and tapering at both ends. It possesses a longitudinal axis bordered on both sides by a series of pigmented leaf-like structures, which give it the superficial appearance of a gill. In this bipectinate type of osphradium the axis is described as an elongated ganglion from which nerves pass off laterally to their terminations in the lamellae. The exact nature of the nerve endings is disputed.

It was first necessary to find a method for rendering the osphradium functionless. It proved to be a very difficult task, principally on account of the inaccessibility of the organ. Since handling of the animal caused more or less contraction of the soft parts, the snail had to be anaesthetized before the osphradium could be reached at all. A concentrated solution of magnesium sulphate in sea water proved to be an excellent anaesthetic for the purpose, as the snails recovered quickly without showing any ill effects. The usual method was to place the animal in the solution in the late afternoon or early evening, where it remained over night. The following morning the operation was performed and the snail returned to an aquarium supplied with running water. It usually became more or less active in several hours and was in condition for study the next day. The olfactory reactions of the snails were always tested before operating, and only those which showed vigorous responses were selected for further experimentation.

Attempts to eliminate the osphradium were first made by scraping it, burning it with a heated platinum wire, or by combining the two methods. Since the elongated organ follows the curvature of the shell, and only about one-half of it can be seen under the most favorable conditions, the task of destroying the deeper portion was largely one of guesswork. The mantle in the vicinity of the osphradium being extremely thin, was easily punctured, or was apt to break through after the operation. When this happened the snail often contracted the mantle and siphon, and accordingly was in poor condition for subsequent tests. A number of individuals, however, recovered from the operation with mantle and siphon externally normal in appear-

ance, and exhibiting behavior characteristic of healthy animals. It soon became evident that some of the snails operated upon responded in the usual way to oyster extract. They followed it about the aquarium, extended their proboscides and ate pieces of oysters. Records of the behavior of ten individuals indicated clearly that they were still scenting food. About as many others treated by the same methods showed a complete, or nearly complete suppression of normal olfactory response.

A suspicion that the attempts at destroying the osphradia had not been entirely successful was confirmed when all but one or two of the entire series of animals were removed from their shells and examined. In every instance a considerable extent of the osphradium was still intact, usually about one-half of it, and always the deeper portion which was not visible at the time of the operation. In nearly all those cases where the organ had been scraped, the mantle showed a hole, usually where the tissue of the osphradium had been destroyed. As before mentioned, the mantle is very thin in the region of the osphradium, and in some instances the injured portion may have been ruptured in removing the animal from its shell. In three individuals whose osphradia had been burned but not scraped, the organs upon examination appeared superficially normal in two cases, and somewhat singed in the third. All three responded characteristically to oyster extract after treatment. One of them, however, failed to show olfactory reactions for over a week after cauterization, although it always ate oysters and otherwise exhibited normal behavior. Response to oyster juice was clearly indicated later.

The fact that the mantle was apparently uninjured when the osphradium was burned was a feature of the results which led to a continuation of the cauterization method. It was evident that the organ should be more severely burned by an instrument which would retain the heat longer than a platinum wire heated over a gas flame. The desideratum was obtained in the form of an electrically heated cautery provided with a handle which permitted control of the current. After considerable experimentation it was found that the deeper portion of the osphradium

could be seen by inserting a small dental mirror into the mantle chamber after the animal had been anaesthetized with magnesium sulphate. This discovery made possible at least the complete elimination of the osphradium without injury to surrounding parts.

The osphradia of four snails, whose olfactory responses had been carefully studied, were cauterized. All four subsequently failed to show scenting reactions when oyster extract was taken into their siphons, whereas they had been decidedly responsive to it before. They also paid no attention to pieces of oyster when offered after testing them with the extract. If, however, a snail was removed from the water, and a piece of oyster placed beneath the head near the false mouth,¹ or in one case, between its lips, the proboscis was usually extended and the oyster eaten. One of the snails also took this food when it was dropped between the head and foot while resting on the side of the aquarium at the surface of the water. Only one test was made with the snail in this position. In short, when a concentrated food stimulus was applied to the skin in the rhynchostome region or to its lips the feeding reaction occurred.

Although highly suggestive, these results were not considered conclusive, as all four animals, at times at least, showed more or less contraction of the mantle or siphon. When, after anaesthetizing three of them, an examination of the osphradia was made, one or two large holes were found in the mantles. Since, at the most, only traces of the osphradia appeared, the organs undoubtedly had been rendered non-functional.

It was evident that the last cauterization method employed was directly injurious to the mantle, and led to undesirable conditions of the siphon. Accordingly, the original procedure of scraping away the osphradium was again undertaken, this time with the aid of a mirror. The lamellae of the organ are delicate and offer little resistance to the flattened tip of a wire,

¹ The so-called false mouth or rhynchostome, which is the opening of the cavity about the proboscis, should be distinguished from the true mouth at the free end of the proboscis. The former is the aperture which is visible on the under surface of the head through which the proboscis is protruded.

the implement used in removing the more inaccessible parts. Nine animals were operated upon and their subsequent behavior recorded. From four of them, however, no final conclusions could be drawn. One had its mantle and siphon so badly contracted that it could not be satisfactorily studied. There was a large hole through the mantle. Another, which failed in several trials to follow oyster extract, or to extend its proboscis, although exhibiting but slight contraction, appeared sluggish and the mantle proved to be punctured. The third snail developed serious contraction of the siphon, so that it could not be adequately experimented upon, and again holes were found in the mantle. The first time it was tested with the extract it extended its proboscis and ate a piece of oyster, and at another time contracted its foot and toppled over. In this position it thrust out its proboscis and then took a piece of oyster. The protrusion of the proboscis in the latter instance was very questionably the result of chemical stimulation, and in the former one, the juices may have been concentrated enough to have effectively stimulated the skin receptors, for the animal was resting. No locomotion was induced by the oyster extract, and when buried in the sand the snail failed to respond to it, although it did so actively before the operation. The fourth animal was one which had shown normal scenting reactions after the osphradium had been partly singed with a heated platinum wire. After scraping away the osphradium, there was no response such as occurred before. In one instance, when the snail was resting against the corner of the aquarium with its foot contracted and its siphon directed upward, the application of oyster juice was followed by an extension of the proboscis, but no locomotion. The position of the snail was such that the stimulating material probably collected over the rhynchostome region and caused the reaction. Abnormal behavior and contraction of the siphon led to an inspection of the mantle where the osphradium had been removed and, as was expected, a hole was found. The view that the ill effects of the operations seen in these and certain other snails were due to puncturing the mantles, which allowed water to pass behind them, rather than to the removal of the

osphradia, is well supported by the condition and behavior of the animals about to be described.

The fragmentary and inconclusive results obtained from four of the snails were in striking contrast to those derived from the study of the remaining five, which were under observation from twenty to twenty-five days after the operations. An examination of the anaesthetized animals with the aid of a mirror after all the tests had been made, showed that the osphradia had been removed without apparent injury to the mantles. The general behavior of the snails was like that of animals which had not been operated upon. They were active in the aquarium, buried themselves characteristically in sand, and there was no abnormal contraction of mantles or siphons. Both morphologically and physiologically they gave every appearance of healthy animals. The earlier experiments demonstrated that the extirpation of a considerable portion of the osphradium produced no observable shock effects, the reactions after the operation being the same as before, and it was exceedingly unlikely, therefore, that the successful removal of the whole of it would do so.

When oyster extract was applied to the siphon tips of these snails no reaction of the usual type ensued. Sometimes the siphon was raised somewhat, or the end flared a bit, but the response so characteristic of a snail scenting food and so clearly shown by these individuals before the osphradia were removed no longer appeared, and not once was the proboscis extended after the application of the extract, although they were tested many times. This cessation of olfactory response was very striking when the snails were buried in the sand. Before the operations four of them were tested once, and one three times in this position. In these seven trials there was not a single failure in the response to oyster extract taken into the siphon. The snails were entirely out of the sand in the maximum time of three minutes, and the average time of less than two minutes after stimulation. After the removal of the osphradia, however, there was complete lack of response under similar conditions. The animal which was given three trials before the operation was tested five times afterward, and the other four were given

three trials each. In each of these seventeen trials the oyster juice was squirted over the siphon tip frequently for ten minutes. The end of the siphon sometimes flared, or was momentarily contracted, indicating a local skin stimulation, but it was quite impossible to induce the animals to leave the sand. That water was being drawn into the siphons in a normal way was determined for each individual by tests with carmine and sea water.

The failure of the snails to respond to oyster extract was closely paralleled by their behavior, while resting or moving on the bottom of the aquarium, when pieces of oyster were given them. Three of them took the food once, but these responses were the only ones recorded in over thirty trials. It was taken three times when the animals were on, or partly on the side of the aquarium near the bottom. After having fed individually over eighty snails upon oysters and noted what often appeared to be an insatiable appetite for them, it was perhaps not strange that this falling off in feeding activity was first attributed to some unrecognizable physiological disturbance resultant of the operations. That this interpretation was incorrect, however, was made evident when the snails were removed from the water and pieces of oyster were dropped under their heads. All five snails ate oysters under these conditions. They also took food, while resting on the side of the aquarium at the surface of the water, when it was placed on their feet close by the head and tentacles. All but one were fed in this position.

Two important facts are brought out by these responses to food. First, the animals were ready to eat, which supports the conclusion drawn from other aspects of their behavior that they were physiologically fit for experimental study. Secondly, as already noted, a concentrated food stimulus is the effective one for causing the feeding reaction when the osphradium is eliminated. The snails without doubt failed in most instances to respond to the food on the bottom of the aquarium because the strength of the stimulating substance was considerably reduced by the rinsing the oyster underwent before it could be directed under the head of the animal, and because of the difficulty in keeping it in contact with the body surface for any length of time.

When the snail was held in the hand, or when it was on the side of the aquarium with its head at the surface of the water, the oyster could be laid upon the skin of the foot and head region with little or no diminution in the strength of its stimulating materials. The point is well illustrated by the following case: One of the snails, which was moving on the bottom of the aquarium, could not be induced to take a piece of oyster which was placed beneath its head. The animal was removed from the water and the same piece, by that time well rinsed, dropped on the under surface of the head. Again there was no response. Finally a fresh juicy piece was taken from a dish and presented to the snail in the same manner. It was taken into the mouth instantly.

The last experiment supports the view that the reactions to oysters are brought about by chemical rather than tactile stimulation. In order to gain more information on this point, a few tests were made as follows: A piece of cotton soaked in sea water was placed against, or close to the under side of the head and the effect recorded. The cotton was then soaked in oyster extract and returned to the same region, and the reaction compared with the preceding one. The snails were held in the hand, or were resting at the surface of the water when the tests were made. In ten trials, including the five animals, the cotton and oyster juice always caused a flaring of the false mouth, or a protrusion of the proboscis. There was no visible reaction to the cotton soaked in sea water except in two trials fifty minutes apart on the same individual, when the proboscis was thrust out. No attempt was made, however, to seize the cotton, whereas the snail plainly tried to take the cotton soaked in oyster juice into its mouth in the two succeeding trials. In three other tests made upon this animal at later times no reaction to the cotton and sea water occurred. Excessive hunger may have been a factor in starting the feeding response in the two instances cited. It is clear that a chemical stimulating agent is the appropriate one for bringing about the reaction to food.

After concluding the experimental work upon these five snails with non-functional osphradia they, with one of the

cauterized animals, which of the four operated upon was in the best condition, were placed in an aquarium supplied with running sea water. No opportunity of observing them again occurred until a year afterwards, when all six were found alive, in fact indistinguishable in their behavior from other individuals of the species. Upon dropping dilute oyster extract into the water in front of their siphons, they responded to it as they had done before the osphradia were rendered inoperative. Their movements were directed by appropriate stimulations with the extract, they extended their proboscides in the characteristic manner, and again ate oysters which were presented to them when on the bottom of the aquarium. The animals were finally anaesthetized and examined, and the fact that their osphradia had regenerated was clearly apparent. Although regeneration of the organs were not complete, several patches of new lamellae covered with pigmented epithelium were present in every instance. Thus with the reappearance of osphradial tissue the scenting reactions returned.

An analysis of the data recorded above leads, in my opinion, to but one conclusion relative to a function of the osphradium. The scenting responses of *Busycon* are dependent on the stimulation of this organ by materials derived from food, the most distinctive feature of which is their diluteness. The osphradium, therefore, is an olfactory receptor.

III. DISCUSSION

1. Movements resulting from olfactory stimulations

Some of the results of the experiments described in the preceding section of the paper require further explanation or discussion. In the first place, however, certain observations upon *Alectrion* should be recorded which were made after the study of *Busycon* was nearing completion. As already mentioned, it has been held by Dimon that the mud snail depends almost entirely on aimless movements for finding its food in water described as quiet. It seemed strange if this were so, since it possesses a siphon and osphradium clearly homologous to those

found in *Busycon*, where the two form an apparatus by means of which the snail under the influence of olfactory stimulation is directed toward food with remarkable precision. A close inspection of the mud snail's behavior in the presence of food showed clearly that the animal did not find it by wandering about until it happened to be encountered. When the snail is crawling, the right and left siphon movements predominate. The organ is not held at the same level throughout its course, but usually the distal end is depressed at the termination of the lateral movement, often so as to touch the bottom, and is lifted as it returns to the opposite side. Frequently it is waved about, is directed upward or even turned backward. The free condition of the siphon in the mud snail thus allows many irregular changes in its position, which may tend to make less evident the right and left ones which in *Busycon*, on account of the restriction of siphon movement by the shell, are directly recognized.

A small piece of butterfish, a little over a centimeter in diameter, was placed on the bottom of a glass aquarium containing several mud snails. The animals after scenting the food found it by movements which unquestionably were directed by olfactory stimulations. They were seen to turn into the fish when headed in a direction which, if followed, would take them by it. Sometimes after circling about, they drew away from the meat, but then proceeded to it in a fairly straight course. All the time their siphons were swung from one side to the other, often being raised between the lateral movements, and their proboscides were extended when they were several centimeters away from the fish. One snail, which scented the food when five to eight centimeters off, circled about rapidly extending and swinging its siphon in the characteristic manner. It then moved toward the fish but, when closest to it, the siphon happened to be directed away, although the organ would have touched the food if it had been swung to the opposite side at that moment. As a result the snail missed the mark by a slight margin and went beyond it about five centimeters, when it turned and took a straight course back to it. Another snail, which was moving in a direction comparable to the last, stretched its

siphon toward the food as it was passing, stopped, extended its proboscis and turned into it. When these tests were made there was no water running into the aquarium to aid the dispersal of the odorous material, and accordingly the responses occurred within a comparatively restricted area. By applying fish extract with a pipette so that it was taken into the siphon when directed to one side, a snail could be made to turn to the right or left at will. It is evident, therefore, that *Alectrion*, although more active than *Busycon*, and possibly more apt to turn from a straight course for reasons not apparent, is nevertheless directed to distant food in the same way as the larger species.

When the olfactory apparatus and the directed responses of the snail are contrasted with those of an animal organized on a plan of more general occurrence, the peculiarities of the former are readily appreciated.

In 1911, Sheldon showed that the dogfish obtains its food principally, if not entirely, through the olfactory sense; and three years later Parker ('14) demonstrated that this fish does not run upon food by accident after scenting it, but finds it through movements resulting from olfactory stimulations which, in part at least, are directed. The latter conclusion is based on the results of experiments upon dogfish with one of their two nostrils occluded. He found that an animal under the influence of olfactory stimulations "can be forced to assume either a predominantly right-handed or left-handed course by occluding the appropriate nostril." The results are discussed in part as follows:

The consistent and striking circular courses that these fishes can be forced to assume have, in my opinion, more than a superficial resemblance to the so-called circus movements of the invertebrates. These movements are dependent on the differences of intensity of stimulation on the two sides of the body and this explanation holds, I believe, for the circular movements of the dogfish. When a dogfish first enters water permeated with odorous material from its food, it invariably makes a quick turn with its head which, if the conditions of the water have been disturbed by currents, is always toward the bait. This movement is followed by other movements of a like kind whereby the fish eventually reaches the bait. When the normal con-

ditions of the fish are disturbed by the complete occlusion of one nostril, the fish swims as though it were in water that was highly charged with odorous particles on the side of its body corresponding to the open nostril and devoid of these particles on the opposite side. The fish therefore turns toward the side of the open nostril, but since, under the artificial conditions of the experiments, this turn does not equalize the stimulus, the motion is continued and a circular form of locomotion results. Thus, in my opinion, the more or less circular movements induced in a dogfish with an occluded nostril by an odorous bait are to be explained upon the same basis as the circus movements of such invertebrates as crustaceans, insects, etc.

Turning now to the snail, it is at once recognized that it has a receptive apparatus concerned with responses to odorous material which, in certain respects, is quite unlike that of the dogfish. Instead of possessing a pair of olfactory organs symmetrically placed on the right and left of the principal axis of the body, it has but one, the osphradium, which is approximately median. Extending forward over the head there is a long tube, the siphon, through which water is conducted not only to the gill but to the olfactory receptor at its proximal end. The more distal part of this siphon is not fixed in position but, when the snail is crawling, is almost continually in motion. For the most part it is moved back and forth from one side of the body to the other, but it may be directed more or less upward and downward and, in the case of the mud snail, even posteriorly. By this provision water and its contained odorous substances can be drawn to the osphradium from various directions.

Let us assume that a whelk is crawling over the sea bottom in the vicinity of a dead fish. From what has been learned of its olfactory reactions under varied conditions, it is possible now to picture mentally the movements which must often occur after the snail scents the food, and to interpret them with some degree of success. As the snail moves, the end of the siphon is shifted from one side to the other, although the foot is carrying the animal in a straight course. Perhaps as the siphon is directed laterally, it happens to enter a stream of odorous material spreading or drifting from the fish, a sample of which passes to the osphradium and stimulates it. The effect is seen in the foot which begins to turn in the direction of the odor. The siphon,

next directed away, fails to receive the stimulating substance, but on the return swing again enters it, the osphradium is again stimulated and the animal moves into the stream of odorous material, swinging its siphon vigorously. The snail continues turning as long as the osphradium is most strongly stimulated by materials coming to it from one side of the siphon's axial position. It may be said to be oriented to the odor stream when the foot is straightened out, and the effective stimulating substance is received by the siphon in its axial position, or when it is equalized on both sides of that position. The siphon continues conducting to the osphradium samples of water and odorous substance in changing concentrations from the region through which it moves; stimulations of the osphradium are followed by foot movements which take the snail toward the place where the strongest stimulating material entered the siphon, until slowly but surely the animal is directed to the source of the scent. The snail, therefore, provided with a water testing apparatus, which may be said to consist of a single receptor associated with a long snout terminating in a shifting nostril, accomplishes what the dogfish does with its paired receptors and fixed nostrils.

When the siphon of *Busycon* is fastened to one side of its axial position, so that it cannot be moved to the opposite side, circus movements occur after olfactory stimulations which are not due to disturbances resulting directly from tying up the siphon. The same form of locomotion may be observed when the organ is given its natural freedom of movement, but is allowed to receive odor only from one side. Under these circumstances the snail continues exhibiting orienting movements, and thus behaves like a dogfish scenting food through a single nostril.

In the case of the dogfish, it was found that olfactory stimulations called forth certain movements described as random ones. Comparable movements, as might be expected, were not observed in the responses of *Busycon*. An animal moving as slowly as this one would be poorly adapted for finding food if,

after olfactory stimulation, it traveled in directions which carried it far from the direct course to the source of the odor.

The arrangement of the olfactory receptors of the fish has at least one slight advantage over the scenting apparatus of the snail. In the fish there is always a receptor on both sides of the head ready to be stimulated, whereas in the snail the osphradium may not be affected by stimulating material present on one side of the body, when it would be of advantage to the animal if it were, because of the fact that at the appropriate moment the siphon may be directed in the wrong way to receive it. This, I believe, is exactly what happened when the mud snail, described a few pages back, first went by the piece of fish meat instead of finding it directly. This defect in the snail's olfactory receptive apparatus, however, under most circumstances cannot be a serious one for a slowly moving animal, and it may be somewhat overcome by the acceleration in siphon movement which usually follows stimulation. In another respect, on the other hand, the snail's scenting apparatus has a distinct advantage over that of most animals possessing paired organs, unless they exhibit greatly accentuated lateral head movements. The area over which a change in the stimulating material can be recorded on the receptor, when *Busycon* is moving in a straight course, is twice as wide as it would be if paired olfactory organs were situated on the sides of the foot, which is about six or seven centimeters broad and ten to twelve centimeters long, and four times as wide if they were on the sides of the head. The siphon 'nostril' of this snail has a side to side range of action which is three times greater than the distance between the paired nostrils of a dogfish about eighty centimeters long, one which is greater than that distance in a large sand shark, and equal to it in a dusky shark of medium size.

2. Taste and smell

Although a detailed study of the sense of taste in the snails was not attempted, some facts pertaining to their gustatory reactions and to the distribution of surfaces sensitive to taste

stimuli were obtained while isolating the responses initiated by olfactory stimuli and localizing the organ of smell. Some of these should be briefly reviewed here.

As Parker and Stabler ('13) have emphasized in a paper on taste and smell, the distinction between the two senses which is most widely applicable is a quantitative one. Olfactory organs are stimulated by relatively weak solutions and gustatory organs only by relatively strong ones. Consequently, when an animal possessing organs of taste and smell encounters a relatively strong food substance, reactions may occur which, under certain circumstances, represent the combined effects of both gustatory and olfactory stimulations. When, however, this substance is sufficiently diluted, a concentration of the stimulating material is obtained which does not affect the gustatory organs, and reactions which it then calls forth are recognizable as olfactory, and the organ stimulated as an olfactory receptor. Since, for example, snails which show easily detectable responses to dilute food materials fail to show them after the osphradium is destroyed, when it is clear that other factors are not influencing the results, the conclusion drawn is that the osphradium is an olfactory receptor. It is equally evident that after the organs of smell have been rendered non-functional, reactions isolated from olfactory ones may be initiated with relatively strong stimulating materials derived from food, and the receptive areas localized. Working, therefore, upon this quantitative basis of distinction between the two senses, the reactions of the snails to food or its derived stimulating juices, as well as the receptive surfaces concerned with them can be determined as olfactory or gustatory.

It is recognized that confusion may arise between taste and the common chemical sense for the reason that relatively strong solutions may stimulate the endings of nerves associated with the latter, as has been demonstrated in a number of vertebrates where the nature of the nerve terminations concerned with the two senses is better known. Since, however, the materials used in stimulating the snails were food and its juices, and reactions occurred which were distinctly positive in character or which

terminated in the obtainment of food, it is reasonable to conclude that external taste receptors were stimulated, and the designation of certain reactions as gustatory is, in my opinion, justified. When the stronger extracts were applied to certain parts of the body such as the mantle edge, a slight local contraction of the stimulated region was the only response noted; the proboscis was not extruded. In such cases there was no conclusive physiological evidence that taste receptors were the ones stimulated.

After the removal of the osphradium, *Busycon* responded to strong stimulating material derived from oysters in contact with certain skin areas by protruding their proboscides and, if food was present, by seizing it, but extract further diluted by its entrance into sea water in nearly all cases in many trials failed to initiate this feeding response. In the one or two instances where the proboscis reaction occurred, the snails were not moving and I believe that the extract, forced from the pipette, settled over especially sensitive skin surfaces in strong enough concentration to stimulate its taste receptors over an extent sufficient to cause the reaction. Snails, however, whose osphradia were operative, habitually showed this reaction in the presence of dilute food juices. The proboscis reaction, therefore, is called forth by either gustatory or olfactory stimuli; in the latter case notably more often when the receptor is under strong or frequently repeated stimulations, and is obviously serviceable in the later stages of obtaining food. The surfaces of the head near the false mouth, the tentacles, and the anterior end of the foot appear to be the external receptive areas most sensitive to the more concentrated stimuli derived from food, although all the regions tested were found to be somewhat so. Taste in the snail, therefore, is a diffused sense as compared with olfaction.

Certain complex reactions, noted particularly in the more active mud snail, now become readily explainable. When fish extract was applied to a tentacle or foot process of a moving snail, the organ contracted and the animal stopped, turned its siphon into the extract, perhaps moved into it, and finally extended its proboscis. When the extract issued from the mouth of the pipette, it was strong enough to stimulate momentarily

the taste receptors of the tentacle or foot; this probably called forth contractions and the sudden movement of the siphon. By the time the extract was taken into the siphon, it was diluted with sea water, but was still strong enough to bring about stimulation of the olfactory organ, which in turn initiated the proboscis reaction. When the greater part of the siphon was cut off, so that the persisting stub failed to reach the diluted extract, the gustatory stimulation alone was in most instances too weak or transitory to bring about more than local contractions, and accordingly the snail continued on its way without protruding its proboscis. Thus when the serial reactions are analyzed, they are seen to be partly gustatory and partly olfactory.

To how great a degree the snails depend upon the olfactory sense in obtaining food has been impressively demonstrated. When very close to it, or even when in contact with it, a hungry animal deprived of the use of its olfactory organ may fail to sense it. No evidence was obtained to indicate that the eyes play any part in food procurement, but the tactile sense may be a factor in it under some circumstances.

In a general way, it may be said that stimulations by relatively weak food solutions call forth locomotion or increase its rate, whereas stimulations by relatively strong ones bring about contractions and cessation of locomotion. Through stimulating the olfactory organ, the former direct the snail to food in the distance, and the latter, by stimulating the gustatory receptors as well, aid in the obtainment of it when at hand; and so, through stimulations by both, the snail procures its food without depending, to any great extent at least, on encountering other forms of stimuli.

IV. SUMMARY

1. *Alectrion obsoleta* and *Busycon canaliculatum* respond to stimulations by dilute food extracts and materials emanating from distant food, which enter their siphons, by increasing the rate of locomotion, or if resting, by moving forward and frequently by extruding their proboscides.

2. Alectrion may crawl with or against a current of water. Individuals, which show more or less tendency to move down stream, move more often against the current when it contains odorous materials derived from food.

3. Since the stimuli calling forth these reactions are relatively dilute chemical ones, the responses are truly olfactory.

4. It was determined by experimental methods that the organ affected by relatively weak chemical stimuli in Alectrion was located somewhere within the mantle cavity. The osphradium is situated on the mantle at the base of the siphon. After the osphradium in Busycon was destroyed the snails failed to respond to dilute food materials, but a year later, when the lamellae of the organ were partly regenerated, the scenting responses returned. The osphradium, therefore, is an olfactory organ.

5. When a snail is moving, the siphon, which conducts water over the osphradium, to the gill, is characteristically swung from one side to the other. In Alectrion this organ is not covered dorsally by an elongated shell siphon, and consequently has greater freedom of movement than in Busycon.

6. When the siphon is directed to one side of its axial position, the foot turns in the direction indicated by the siphon, if it conducts odorous particles which stimulate the osphradium. The snail may be said to be oriented to an odor stream when the foot is straightened out and the effective stimulating substance is conducted to the osphradium by the siphon in its axial position, or when the stimulus is equalized on both sides of that position. Accordingly, a snail can be led about an aquarium by squirting dilute food extract over the end of the siphon when it is pointed in the direction it is desired the animal shall take.

7. When two cheese cloth packets, one containing a piece of oyster, are fastened in front of and lateral to the distal end of the siphon, one on the right, the other on the left, the snail circles toward the source of the strongest odorous substance coming from the oyster, that is: to the right when the baited packet is on the right of the siphon tip, and to the left when

it is on the opposite side. Similar circus movements take place when the siphon is fastened to one side of its axial position, and oyster extract is squirted into the water in front, and to the right and left of the snail.

8. The snails do not find food by coming upon it accidentally, but are directed to it by movements brought about through stimulations of the olfactory organ with odorous substances conducted to the receptor in varying concentrations by the moving siphon. By means of an olfactory apparatus, consisting of a single organ of smell associated with a siphon terminating in a shifting 'nostril,' for sampling the surrounding water and its contents, the snail is as successfully directed toward distant food as an animal which, like the dogfish, possesses paired olfactory organs and fixed nostrils.

9. All the skin surfaces of the snails which were tested were found to be more or less sensitive to the more concentrated stimuli derived from food.

10. When fish extract is applied in its full strength to a tentacle or anterior foot process of a moving Alectrion, the animal stops and turns its siphon into the stimulating substance. Busycon deprived of its osphradium often fails to give evidence of sensing food which is placed near it, or perhaps in contact with it. When, however, the snail is removed from the water, or is resting on the side of an aquarium at the surface of the water, it characteristically protrudes its proboscis and takes food placed on the anterior end of the foot, or in contact with the head and tentacles. These positive reactions of Alectrion and Busycon are the effects of stimulations by relatively strong food solutions and, therefore, are gustatory. The principal external receptive areas for taste stimuli appear to be the tentacles, the anterior end of the foot and the under part of the head.

11. The snails obtain food through the senses of smell and taste, but the latter is effective only in the last stages of its procurement. There is no evidence that the eyes play a part in the reactions to food, but the tactile sense may do so under some circumstances.

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THE PHYSIOLOGY OF ASCIDIA ATRA LESUEUR¹

I. GENERAL PHYSIOLOGY

SELIG HECHT

FIFTEEN FIGURES

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I. INTRODUCTION

Ascidia atra is a large tunicate common in Bermuda and the West Indies. It was first described from Guadeloupe in 1823 by Lesueur. Many years later it was found in Bermuda by the Challenger Expedition, and figured by Herdman ('82), who, however, confused it with the European form *Ascidia nigra* Sav. The distinction between the two is that the European species possesses intermediate papillae on the longitudinal bars of the branchial sac, while the American species does not (VanName, '02).

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Ascidia atra lives attached to rocks, in most cases well under low water. The attachment of the larger animals is by the posterior edge; in the smaller individuals a portion of the left side is frequently attached as well. The species is well distributed throughout the Bermuda Islands. Although animals for this research were obtained from many regions, the main supply came from localities very near Agar's Island.

Ascidians first became of interest during the last half of the nineteenth century. Their significance in relation to the origin of vertebrates, which was first made apparent by the work of Kowalevsky ('67), resulted in innumerable researches on the anatomy and embryology of the group. Since that burst of activity, sixty years ago, the knowledge of ascidians has not kept pace with the newer points-of-view. As a consequence, little, indeed, is known of the life and activities of these animals. It is with the hope of supplying this deficiency that the present series of papers is presented.

The work was done at the Bermuda Biological Station with the assistance of a grant from the Humboldt Fund. I wish to acknowledge the kindness of Prof. E. L. Mark, who made this grant possible, and at whose invitation the experiments were performed in Bermuda. To the Resident Naturalist, Dr. W. J. Crozier, the depth of whose friendship was manifest in many ways, I extend my frank admiration and thanks.

My chief indebtedness, however, is to Prof. G. H. Parker. His teachings and researches, which have the "rare merit of combining both anatomical and physiological view-points," have influenced my work and thought. It is a privilege to express my gratitude for the inspiration which he has given me.

II. EXTERNAL APPEARANCE

1. *Orientation*

At the outset of this account of the physiology of *Ascidia atra*, it is necessary to define the various surfaces and planes of the body. The earlier writers on ascidian anatomy were far from agreed on the application of such terms as anterior, poste-

rior, dorsal, and ventral. As a result, however, of the embryologic investigations of his time, Kupffer ('75) applied these designations correctly. The nomenclature suggested by him has been accepted by all of the later workers (Herdman, '82), and will be used in the description of the present species.



Fig. 1 Medium sized specimen of *Ascidia atra*, life size, showing view of right side.

The large opening of the animal is within the rim of the oral siphon, and the smaller one is within that of the atrial siphon. These two openings are at the anterior end, the oral being toward the ventral edge, and the atrial toward the dorsal edge. The place of attachment is at the end opposite the siphons, and constitutes the posterior part (fig. 1). Consequently, the side of the animal which shows many creases and irregularities in the test is the right side, and the smooth face forms the left side.

According to this scheme, the pharynx, or branchial sac, of *Ascidia* covers the right side almost completely, whereas the renal body and the intestine lie on the left side. The atrial cavity extends along the dorsal edge and terminates anteriorly in the atrial siphon. Quite unusually for this genus, the heart lies on the right side along the ventral edge.

2. Color

On first sight *Ascidia atra* appears to be of a dead black color. Closer examination shows that it is really a very deep blue. The color is located in the test, on the inner surfaces of the siphons, and on the outer faces of the oral tentacles; it may even extend beyond the tentacles into the anterior part of the branchial sac. In sections of the animal, the blue pigment is shown as a thin rim marking the outer edge of the test.

The coloring matter is insoluble in water. Acetone extracts of the test are reddish in appearance, and do not resemble the opaque purplish blue seen in sections of the test. The extract has the properties of an indicator; it is red with acids and green with alkalies, the color change occurring near the neutral point (Crozier, '16).

The blue pigment is contained in spherical granules, which are nearly all of the same size: approximately 3 micra in diameter. They occur in very compact groups of four to six. Although each group may represent a cell containing the pigment granules, it is difficult to make out any cell substance or cell boundaries in thin sections of fresh and fixed tissue. Therefore, it seems improbable that the granules as they are found in the test are within the living substance of a pigment cell.

The groupings may, however, represent the remains of metamorphosed cells whose cytoplasm has disintegrated. On the basis of such an idea, there should be present in the body of *Ascidia* some living cells which would be the precursors of the pigment groups; and, moreover, it should be possible to find intermediate stages between the two.

It was not difficult to satisfy the first of these requirements. The blood contains cells whose volume is the same as that occupied by the compact group of pigment bodies. In addition, these blood cells are packed full of spherical granules whose size and number correspond to the pigment granules in the test. Most of the blood cells are of a rich green appearance, the color being resident in the granules. There are also to be found, in much less abundance, similar blood cells whose granules instead of being a transparent green, are an opaque, dark blue, which to all appearances is identical with the color of the test. It was possible to observe the change from green to blue in individual cells in drawn blood under the microscope. I considered it, therefore, extremely likely that the blue blood cells, representing the later stages of the green cells, are the forerunners of the groups of pigment granules in the test.

In order to prove this satisfactorily, it was necessary to find a stage between the free, blue cell and the group of pigment granules imbedded in the cellulose of the test. Examination of sections of the test brought out only a few, and these doubtful, instances, indicating that pigment deposition in an adult *Ascidia* is probably not a very active process.

The evidence came when it was found that an animal would regenerate its test. An individual denuded of a portion of the test began almost at once to secrete a new one. At the end of one day, a thin layer of cellulose of the characteristic color had been formed over the denuded portion. When this delicate layer was removed and examined with the microscope, there were found hundreds of definitely shaped, blue blood cells imbedded in the cellulose, imparting to it the usual color of the test. As a result of this, it seems safe to conclude that the blue pigment granules in the test of *Ascidia* are the remains of the metamorphosed green cells of the blood.

In this connection the observations of Caullery ('95) are of interest. *Botrylloides cyanescens*, which in nature is yellowish green, turns blue after remaining in the laboratory. Caullery found that the green color was due to cells which contained a number of colored granules, and that the blue appearance in

captivity was the result of the change of these granules to a deep blue. The figures which he gives (Caullery, '95, fig. 52) for these cells resemble the blood cells of *Ascidia* and of ascidians in general (Cuénot, '91). The color change in *Botrylloides* is artificial; in *Ascidia atra* it is in the regular course of events.

3. Formation of the test

Freshly collected specimens of *A. atra*, as well as animals in their natural surroundings, possessed a bright and clean appearance, which was often lost in the laboratory in a short time. In confinement, the outer surface of the test soon changed to a dull gray. The gray material was gradually sloughed, coming off in shreds, and resembling a human skin peeling after a sunburn. Although the layers were removed by the movement of the water, more appeared in a short time, and the animals continued to shed the outer portion of the test as long as they remained in the laboratory.

The animals were kept in 'battery jars of about ten liters capacity, into which the seawater flowed in a gentle stream. Under such circumstances, the water surrounding the animals had but little motion. This is quite in contrast to the comparatively turbulent conditions to which the species is normally subjected. Consequently, it seemed probable that the appearance of the test was merely a superficial laboratory product, and not due to any real effect on the animal. Indeed, individuals kept in smaller jars, in which a more vigorous current was present, showed little sign of this surface change. The sloughing, therefore, merely indicates that *Ascidia* renews its test continually by secreting fresh test material on the inside, and allowing the outside surface to disintegrate and to be removed by the action of the waves.

This conclusion is strengthened by the phenomena which attend the regeneration of the test. Occasionally animals were collected which showed an appearance that can be interpreted only as a regeneration of the test and perhaps of other structures (Hirschler '14). Figure 2 is a sketch of such an individual.

The test shows a ragged surface undoubtedly representing the places where it had been torn. Such a test is highly instructive. In cross section (fig. 2) it can be seen that regeneration had not taken place by the mending of the injured edges, but by the growth of the new test around the body tissue. It does not seem as if the injured portion had been specially reconstructed; but rather as if the test material had been secreted by the surface generally, and only incidentally covered the injured part.

These appearances may be duplicated experimentally. Ascidians present varying degrees of ability to regenerate the test. Some, like *Cynthia* and *Phallusia*, seem incapable of surviving

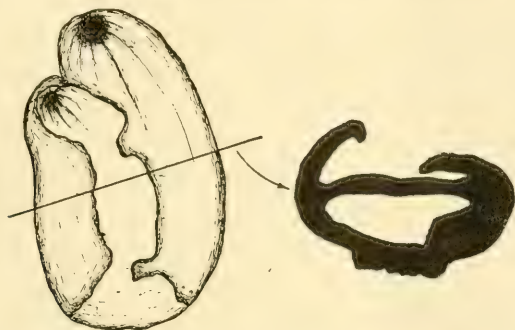


Fig. 2 Sketch of regenerated animal. The transverse section of the test shows the mode of regeneration.

even partial removal of it (Fol, '08). *Ascidia atra*, however, not only recovers rapidly after portions of the test have been removed, but it makes good such deficiencies in a short time.

It has been described how, within a day after the removal of a section of the test, there has already been secreted a thin layer of cellulose. This film is continuous with the innermost surface of the uninjured test, and adheres closely to the soft tissue which has formed it. Animals in this condition, when placed in a sheltered position in the natural environment of the species, continue to thicken the film until it assumes the ordinary dimension of the test. In one animal, for example, a hole several centimeters square was mended in two weeks. Sections of such tests are identical with those of animals like the one in figure 2.

The edges of the old test gape at the injured region, and the new test which is formed underneath them is continuous with the old test where the two meet close to the mantle tissue of the animal.

It was impossible to secure complete regeneration in an animal whose entire test had been removed. This was due solely to a deficiency in technic, and not to an incapacity on the part of the animal. The seawater supplied to the laboratory contains almost no plankton organisms, and since these furnish the food supply of *Ascidia*, even perfectly normal animals died after a week or more of laboratory confinement. I was unable to devise a method of keeping animals without a test out in the open water, because they could not be attached to anything. Consequently it was possible to observe them only for a few days in the laboratory. Here they showed the usual beginnings of regeneration. After a day, a thin layer of test material was formed on the right side of the body and on the entire surface of the siphons, but not on the surface of the renal body. Soon the cellulose began to extend over to the left side, making the bare region smaller and smaller. Undoubtedly, under better conditions, a complete test would have been regenerated.

The newly formed test material is pigmented in the usual way. The pigmentation is not dependent on the presence of light. Animals whose tests had been removed at night, were kept in complete darkness for several days. They regenerated as usual, and the fresh test contained the blue pigment. Moreover, a new, pigmented test will form on the right face of the animal under the intact, opaque, old one, when the latter has been accidentally separated from the ectodermal surface which secretes it. Therefore the formation of a pigmented test is not the result of a photic stimulus only.

These regeneration experiments, as well as the phenomenon of sloughing, indicate that normally there is a continuous addition to the thickness of the test, in order to compensate for the disintegration of the exterior, and for the changing size of the animal.

4. *Amphipod commensal*

One of the striking things associated with the external appearance of *Ascidia* is the frequent presence of the young of a species of *Orchestia* within the cavity of the oral siphon. The occurrence of crustacean messmates in ascidians has long been the subject of comment, and many species of copepods have been described for ascidians all over the world (Scott, '07). However, with the probable exception of Verril's ('70) report of an amphipod in the 'interior' of *Ascidia callosa*, I have found no previous record of the free association of an amphipod in the branchial cavity of an ascidian.

The species in *A. atra* is a pretty animal varying in size from two millimeters to nearly a centimeter. It possesses bright red eyes and a dark band across the middle of the back, both structures showing conspicuously against the whiteness of the body.

In the oral cavity of an *Ascidia* which has not been disturbed for a time, the amphipods are arranged near the rim of the siphon with the anterior end facing outward. Frequently as many as ten may be found in this position in a single siphon. It is a startling sight, when the blackness of the interior of the siphon is illumined, to see the brilliant red eyes of the creatures arranged in a circle a few millimeters within the cavity.

The amphipods are capable of rapid locomotion when forced to leave their host, and may perhaps be free living at times. Their position in the oral siphon of *Ascidia*, however, is of distinct advantage to them. The water current entering the oral siphon brings with it a host of small organisms to serve as food for *Ascidia*. The amphipods share this with their host, and, therefore, furnish an example of real commensalism.

III. FOOD AND ENERGY SUPPLY

The only source of metabolic and growth materials which is available to *Ascidia* is the surrounding seawater with its suspended and dissolved content. In order to utilize this supply, the animals perform certain activities whose function it is to furnish quantities of fresh seawater continuously, and to remove

therefrom the substances necessary for the existence of the species. Both of these processes are accomplished in the branchial sac. The enormous development of this structure testifies to the necessity of working on a large scale in order to abstract the relatively meager proportion of food and energy contained in the seawater.

1. Water current

A study of the water current (Hecht, '16) has already shown that this form of activity has the following properties. The current is produced by the cilia of the branchial sac. It is maintained under a low pressure of 1.7 mm. of seawater. The quantities of water moved are large; in a medium sized individual, 173 liters of seawater are transported in a day. The volume of water moved per unit body weight varies inversely as the size of the animal.

Since the water enters by way of the oral siphon, and leaves through the atrial siphon, it is of primary importance to *Ascidia* to avoid a mixing of the incoming and outgoing currents. In the open water the movements of the sea undoubtedly change the water immediately surrounding an individual, so that a fresh supply of seawater is frequently available. *Ascidia*, however, does not rely on such a chance renewal of its food and energy supply, because even in very quiet water, such as that in a large dish in the laboratory, the two water currents are definitely isolated from each other.

If, in such a dish, particles of carmine are floated near the atrial and oral siphons, it is at once apparent that the outgoing current is considerably stronger than the incoming current. Figure 3 shows a drawing of a small specimen of *Ascidia* life size. The arrows near the oral siphon indicate the range of its activity, that is, the distance from the opening within which a particle of carmine was sucked into the cavity. For this specimen the distance was at most 5 millimeters. The arrow pointing away from the atrial siphon represents the distance within which a particle was deflected by the outgoing current. This range was ca. 65 millimeters. Other individuals showed similar relations

for the range of activity of the two currents. In columns 2 and 3 of table 1 are given the values obtained for two additional animals. Record VI.20.3 is of an individual about twice, and VI.22.1 of an individual about four times, the size of the specimen in figure 3.

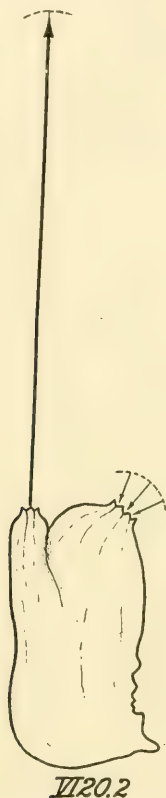


Fig. 3 Range of the incoming and outgoing streams of the water current.

The maintenance of such a contrast in the force of the two branches of the water current is accomplished by a difference in the size of the openings of the oral and atrial siphons. The cilia furnish the motive power, and the same quantity of water which they move passes through both openings in the same time.

TABLE 1

| ANIMAL | RANGE | | DIAMETER | | ANGLE BETWEEN SIPHONS |
|---------|------------|------------|------------|------------|-----------------------------|
| | Incoming | Outgoing | Oral | Atrial | |
| | <i>mm.</i> | <i>mm.</i> | <i>mm.</i> | <i>mm.</i> | |
| VI.20.2 | .5 | 65 | 4.0 | 2.5 | 43° |
| VI.20.3 | 12 | 110 | 5.5 | 3.5 | 65° |
| VI.22.1 | 15 | 230 | 13.0 | 6.0 | 35° |

The oral orifice is large, whereas the atrial is small. Therefore, the velocity, and consequently the momentum, of the water in the atrial current is greater than in the oral current. The diameters of the siphon rims of the three animals mentioned are given in columns 4 and 5 of table 1. The figures are not very accurate, because of the difficulty of maintaining the living animal in a constant state of expansion. They show unmistakably, however, that the difference in the force of the two currents depends, in the main, on the size of the siphon orifices.

A second significant factor concerned with the separation of the two currents is the angle formed by the diverging axes of the two expanded siphons. In the last column of table 1, this angle is recorded for the same three animals. The individual variation in the extent of the divergence of the siphons is surprising; the net result, however, is that the currents are prevented from mixing. The combination of a difference in range with a difference in direction of the two streams of the water current makes *Ascidia* independent of the fortuitous movements of the surrounding sea.

2. Feeding

The stream of seawater which passes through the branchial sac of *Ascidia* brings with it a supply of solid food in the shape of plankton organisms. The exact method which is used in the collection of these organisms has been the subject of conflicting statements.

Earlier writers, such as Roule ('84), described it as follows. The mucus secreted by the endostyle is spread over the inside of the branchial sac by the ciliary activity of the gill bars. This

mucus catches the food particles which come in with the water, and the mixture of food and mucus is transported across the face of the branchial sac, dorsally and posteriorly into the oesophagus. In recent textbooks (Herdman, '99) the process is described in a totally different way, somewhat like the following. The mucus from the endostyle passes anteriorly to the peripharyngeal grooves. Here the food particles are caught at their very entrance to the branchial sac, and carried by the mucus on its way along the dorsal lamina to the oesophagus. Delage et Herouard ('98, p. 144) point out the differences in these descriptions, but cautiously avoid anything but a generalized account of feeding.

The matter has been recently investigated on many transparent ascidians by Orton ('13), who has proved very clearly that the earlier accounts are correct. I have examined the process of food collection in *Ascidia* and in the transparent *Ecteinascidia turbinata*, and my observations are in complete agreement with those of Orton. Occasionally specimens of *A. atra* are found which are quite translucent. By feeding carmine to such animals, it is possible to see the red band of mucus-entangled material swept along the branchial sac, upward and backward into the oesophagus.

The mechanism by means of which the transportation is accomplished deserves a closer scrutiny. At regular intervals along the junctions of the transverse and longitudinal vessels of the branchial sac, there are present small papillae which project into the cavity of the sac. A papilla is really the wall of a blood sinus, and in *A. atra* its ventral wall is composed of a ciliated epithelium (fig. 4, *A*). At its junction with the intersecting vessels, I have always found a flat semicircle of what seems to be smooth muscle cells (fig. 4, *B*). The location of the ciliary surface of the papilla and the muscle at its base are intimately concerned with the collection and movement of the food.

By removing a part of the test and branchial sac, it is easy to observe with a binocular microscope the function of the papillae. Food particles which are filtered by the meshes of

the branchial sac are rapidly lashed to the tips of the papillae by their ventral cilia. Here they are caught by the mucus, and incorporated into the thread of food which is passing across the branchial sac. This cord of mucus and food is transported by the papillae. Waves of contraction bring two rows of papillae together, and by the action of the cilia the food cord is passed from one row to the next, until it reaches the oesophagus.

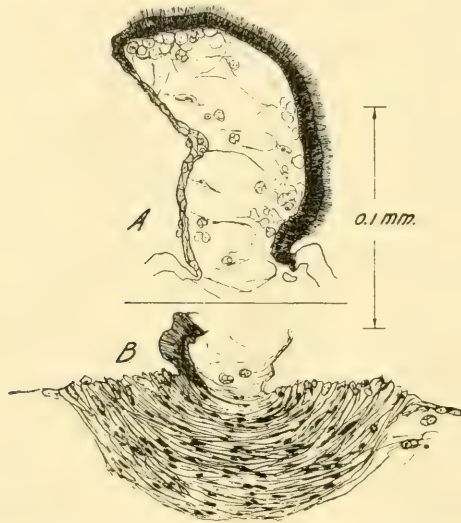


Fig. 4 Papilla of the branchial sac. *A*, median section; *B*, section at the base of the papilla.

The mechanism for these papillary movements is probably local, because touching a papilla with a glass rod causes a contraction to appear. This would indicate that the waves are the result of a series of stimulations of the papillae by the contact of the food mass.

The food as it enters the oesophagus is in the shape of a cord, and in this manner it is passed along the digestive tube. With the food also goes the mucus. Although the food is digested and absorbed, the mucus is probably not affected at all. When

the feces come out in neat, flat, oblong packets, they are incased in a thin layer of gelatinous material, which is probably the mucus. The presence of the gelatinous covering of the feces is best seen in animals which have been in the laboratory for some days. In such cases the feces contain but little excrement, and are composed mainly of a transparent mass of mucus.

The seawater in the laboratory is coarsely filtered, and contains very few organisms. In this way the food supply of *Ascidia* is cut off; consequently, it does not live long in confinement. This shows that the dissolved organic content of seawater, to which Pütter ('07) has attributed such great importance, is of little significance in maintaining the metabolic balance of *Ascidia*. The species has developed an elaborate mechanism for capturing the organisms in seawater, and without them it slowly starves to death.

IV. THE MOVEMENTS OF ASCIDIA

Ascidia atra, though permanently attached to the rock, is capable of moving not only certain of its structures, but also of bending and contracting its body in relation to its base of attachment. The siphon rims can close and open, the body can contract along the dorso-ventral axis, and the entire animal can bend with a surprising degree of vigor. When arranged in certain combinations and sequence, these activities form the reflexes with which the animal responds to stimulation. From such an aspect they will be considered in the description of the sensory reactions of the species. At present, however, my object is to present the physiology of these movements in themselves by examining the factors which are concerned in their production.

1. *Siphon rim closure*

Ascidia is usually described as possessing eight lobes on the rim of the oral siphon and six on the atrial. These are shown in figure 1. All such photographs and descriptions are of dead animals and tell only a partial truth. In the normal, living animal under water, these lobes are not shrunken and collapsed,

but stand out expanded on the siphon in the form of thin lappets (fig. 5).

Under special circumstances it is possible to secure a local contraction of the region near an individual lappet. Ordinarily, however, the entire siphon rim shuts as a unit. This closure is



Fig. 5 Sketch of living, expanded Ascidia, to show the cheeks on the right side and the protruding lappets on the siphon rims.

conditioned by the presence of well-defined ridges and folds in the test, along which the contraction takes place. An end-on view of a nearly closed oral siphon (fig. 6) shows that the alternation of folds and ridges depends on a surprisingly accurate pattern, which involves thick and thin portions of the supporting test.

The closing of the siphon rim, however, is more than a mere puckering together of its edge due to the action of circular muscle fibers. The rim is not only pulled together, but is also drawn down toward the body of the siphon. This is due to the action of longitudinal muscle fibers which, in the siphon, lie nearer the cavity of the siphon than do the circular muscles.

The siphon rim in *Ascidia* is so opaque that it was impossible actually to observe the action of the two sets of muscles. A transparent species *Ecteinascidia turbinata*, furnished the desired opportunity. Individuals two or three days old, measuring three to four millimeters in length, can readily be examined

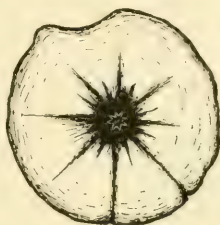


Fig. 6 End-on view of a partially closed oral siphon, showing the geometric arrangement of the folds in the test. The left side of the body is uppermost.

with the low power of the microscope. These animals show the two factors of siphon closure beautifully. At first the circular (sphincter) muscles contract and partially close the rim. This is followed by a contraction of the longitudinal fibers, which results in a drawing in of the rim, thereby completing the closure. In these young individuals I have frequently observed the longitudinal muscles of the oral siphon contract so vigorously that the upper portion of the siphon was completely inverted and tucked into the branchial cavity. In *Ascidia* this retraction is provided for by a sudden decrease in the thickness of the test near the rim (fig. 10). The combined action of the two sets of muscles results in a closure which is really complete. No trace of a water current can be demonstrated after the siphons have been shut.

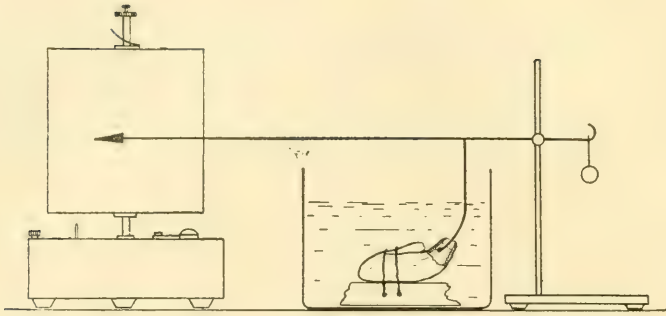


Fig. 7 Apparatus to record the movements of Ascidia.

In order to analyze the movement of the siphon rims, I secured kymographic records of their activity. The apparatus which was employed is represented in all essentials in figure 7. The drawing needs little explanation. The long, light, aluminum lever was nearly, but not quite, balanced by the weight on the short arm. This slight excess on the long arm kept the pendant vertical rod in continual contact with the right edge of the siphon rim. Such a procedure proved more effective and less disturbing to the animal than actually attaching the lever to the tissue. In addition the vertical arm was curved so as to fit into the cavity of the siphon. To keep the animal in place, it was fixed to a piece of plate glass, which was heavy enough so that even a vigorous movement of the entire animal did not change its position.

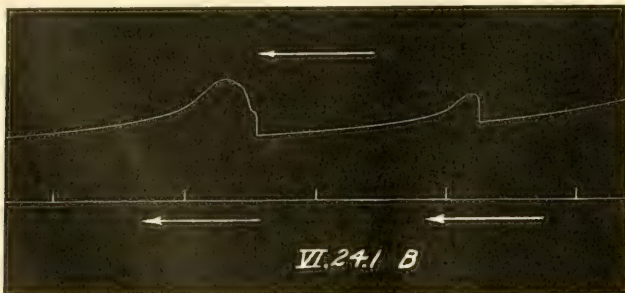


Fig. 8 Record of siphon rim contraction, and record of body bending. The base line marks one minute intervals.

The smaller contraction in figure 8 represents a siphon rim movement; all the other records are almost identical with it. Curve 7 in figure 11 is another example, and represents in addition an analysis of the movement. The record is divisible into four distinct phases. The first two are the phases of contraction; the second two those of recovery.

The first phase is an almost straight line, and represents, therefore, a comparatively rapid contraction of the siphon rim. Although of short duration, lasting about a second, this portion of the movement accomplishes nearly three-fourths of the total contraction. The remaining closure is made at a much slower rate, as is shown by the amplitude and duration of the second phase. The condition of maximum contraction is reached in approximately four seconds. Recovery begins almost immediately, and in the beginning is comparatively rapid. The third phase of the record may be defined as that portion which lies between the position of maximum contraction and the point where the curvature of the line changes so as to be convex to the time axis. It lasts nearly twice as long as the second phase, and includes about one-half the recovery of the siphon rim. The last phase of the movement is the longest, occupying nearly three-fourths of a minute. At the end of it the siphon rim has assumed the diameter which it had at the beginning of the contraction.

An analysis of the time relations of the phases of two separate records of the same siphon rim is given in the accompanying table (table 2). The two movements were made under the same conditions within a few minutes of each other, and were produced by the same intensity of mechanical stimulus. The similarity in the resulting records is very evident.

TABLE 2
Siphon rim closure. Exp. VI.23.1

| | DURATION OF PHASES, SECONDS | | | |
|----|-----------------------------|-----|-----|------|
| | 1 | 2 | 3 | 4 |
| I | 1.1 | 3.4 | 5.3 | 36.8 |
| II | 1.5 | 3.4 | 9.2 | 40.5 |

It will be noticed that the general shape of the curves produced by the closing and opening of the siphon rims resembles that of the contraction and recovery of smooth muscle (Winkler, '98). The effective agent in the closure is, indeed, the sphincter of smooth-muscle cells in the siphon working against the elasticity of the tissues and the test. Although the presence of the test undoubtedly helps in the opening of the rim, the recovery from the contracted condition can occur without the test. Animals

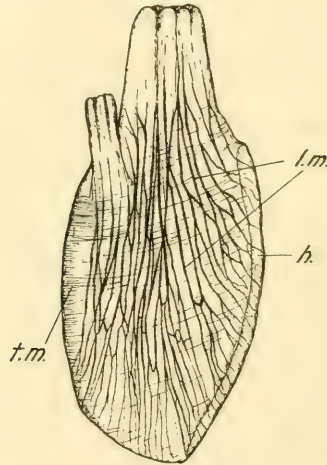


Fig. 9 Right side of animal with the test removed. *h.*, heart; *l.m.*, longitudinal muscles; *t.m.*, transverse muscles.

from which the test had been removed were still capable of closing and opening the siphons. It seems reasonable to suppose that the elasticity of the tissues which is responsible for this recovery is due to the presence of spaces filled with blood under pressure.

2. Body movements

The bending of the body on its long axis occurs so that the right side of the animal always forms the concave surface of the bend. This is accomplished by the contraction of longitudinal muscle strands which lie on the body wall of the right side only

(fig. 9). In the living animal this side of the body adheres firmly to the test. The left side, however, which includes the renal organ, parts of the intestine, etc., is free from the test, the two being connected only by a single blood vessel. Consequently any contraction of the long muscle strands on the right side will result in a bending of the body and test in that direction. This is facilitated by the fact that the right side of the test is much thinner than the left (fig. 10).

The bending of the body on its long axis is always associated with a movement of the siphon rims. This becomes clear when



Fig. 10 Longitudinal section of test, passing through oral siphon.

its kymographic record is examined (fig. 8). The kink in the very first part of the curve denotes a siphon rim closure. It will be seen that the movement of the siphon precedes the vigorous activity of the body as a whole.

The curve made by the bending of the body (fig. 11, curve 6) resembles in all essential features the one made by the siphon rim contraction (curve 7). It may be divided into four phases, the durations of which are relatively the same as in the activity of the siphon. The first phase is short, and accomplishes the main extent of the contraction. During the longer, second, phase a slower activity brings about the maximum point in the curve. The resumption of nearly the normal position of the body is accomplished in the third phase, whereas the complete relaxation

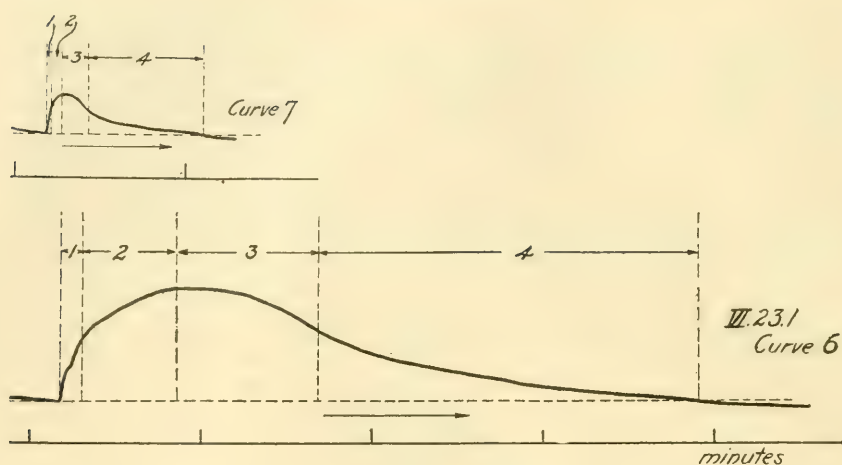


Fig. 11 Analysis of records. Curve 7, siphon rim closure; curve 6, body bending.

takes place slowly during the much longer period of the fourth phase. The actual time occupied by the different periods is given in the accompanying table. In table 3 there is represented an analysis of three records of the movements of one animal produced under the same conditions and intensity of stimulation.

The appearance and duration of the second and third phases of the record depend in the main on the intensity of the stimulus which produces the body contraction. The more intense the stimulus, the longer do the two phases last, their greater duration showing itself in the maintenance of the condition of maximum contraction. Compare, for example, the values in table 3 (also fig. 8) with curve 6 of figure 11. Although both were

TABLE 3
Body Contraction. Exp. VI.23.1

| | DURATION OF PHASES, SECONDS | | | |
|-----|-----------------------------|------|------|-------|
| | 1 | 2 | 3 | 4 |
| I | 7.3 | 19.4 | 26.3 | 128.9 |
| II | 6.8 | 17.3 | 23.6 | 143.5 |
| III | 6.8 | 16.8 | 18.4 | 96.8 |

obtained from the same individual, curve 6 is the result of a stimulus twice as intense as those whose records are given in the table, and consequently, the two phases last considerably longer.

Whereas the actual bending of the body involves merely the action of the longitudinal muscles, the recovery to the normal shape depends upon several factors. One of these is the activity of a set of muscles situated on the left side of the siphons. Although these muscles are not well developed, and extend for a short distance only, they act probably like extensor muscles, and tend to antagonize the action of the long muscle strands of the right side. A second factor is the elasticity of the soft tissue of the body. That these two agencies alone are capable of bringing an animal back to normal shape is shown by the complete, though slower, recovery of animals with the test entirely removed.

The test, however, is a structure of considerable significance in the resumption of the normal form after the bending of the animal. Although apparently homogeneous, the cellulose material of ascidians has been shown to possess a fibrillar structure visible in polarized light (Schulze, '63). Probably this heterogeneity, which is also to be seen in some stained preparations of *A. atra*, is of importance for the elastic properties of the test. Its peculiarities resemble those of a viscous solid. To a sudden distortion, the test will respond in a manner comparable to most elastic bodies. If, however, it is subjected to a slow distortion, it will partially accommodate itself to the new form, and never return to the original shape.

In the bending of *Ascidia* the activity is sufficiently rapid to cause an immediate elastic recoil on the part of the test. Sections of the body show that the resilience of the test is utilized to good advantage (figs. 10 and 12). The left side is noticeably thicker than the right and, consequently, serves as an elastic back which antagonizes the muscles of the right side. Although controlled by the relaxation of these muscles, the elastic rebound of the left side probably serves in a large measure to straighten the curved body.

The difference in the elastic response of the test to strains of short and long duration may be the explanation of the distorted appearance of many laboratory and museum specimens. The collection and transportation of living animals involve a continued stimulation. This results in the maintenance of the curved condition for a long time, until finally the elastic limit is passed, and the animal remains permanently abnormal in appearance.

The elasticity of the test is further made use of in the third type of movement of which *Ascidia* is capable. This is a contraction of the body along its dorso-ventral axis, in such a way that the right side forms the concavity. The muscles which are concerned are the transverse fibers on the right side (fig. 9). In order that they may exert their influence, the test is thinned

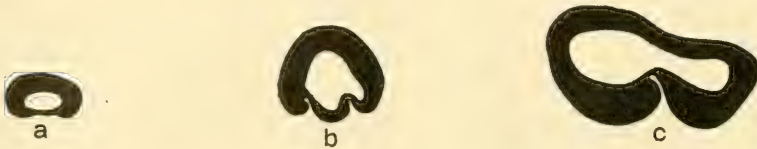


Fig. 12 Transverse sections of test. *a*, near tip of oral siphon; *b*, near base of oral siphon; *c*, through middle of body.

out along a narrow line, in the middle of the right side, running parallel to the long axis. The contraction of the transverse muscles bends the test along this line, with the consequent formation of two prominent cheeks (figs. 5 and 12c).

The contraction of the body on its dorso-ventral axis extends in some cases well into the oral siphon. There are circular muscles present in the siphon, which by their contraction can decrease its diameter. Here also the test shows an arrangement of thick and thin portions on the right side, whereas the left side still maintains its uniform thickness in order to aid in the recovery (fig. 12b).

It is consequently obvious that the test is intimately concerned in the contraction of the body on its short axis. The same is true for the siphon rim movements and especially for the bending of the body on its long axis. Definite structures in the test go

with definite sets of muscles. Therefore, besides serving as an excellent covering for the soft internal parts, the test in addition functions as an exoskeleton, on which depends the proper execution of the movements of the animals (Fol. '08).

3. *Spontaneous movements*

In the continued observation of *Ascidia* under all sorts of conditions, it became evident that complete movements of the body and siphons often occurred when no apparent external stimulus was present. The animal is extremely sensitive to mechanical stimulation, and at first I was inclined to attribute these aberrant contractions to very slight movements of my body or of other people in the laboratory. Such an explanation was, however, abandoned when the same movements occurred under conditions which precluded this source of stimulation.

Somewhat similar spontaneous contractions have been described for the cirri and oral hood of *Amphioxus* as the result of the mechanical stimulation of the cirri by the accumulation of particles of sand (Parker, '08, p. 431). This explanation does not hold in the case of *Ascidia*. When animals which had been carefully washed were placed in filtered seawater, they continued to perform spontaneous movements. Moreover, animals with the test entirely removed and with the greater part of the siphon cut away, and consequently deprived of their sensory apparatus, still exhibited frequent contractions. The factors for their production, therefore, rest within the organism itself.

The solution of the difficulty came when a record was kept of the appearances of the spontaneous contractions under conditions which excluded external stimulation. Several animals were placed in individual battery jars containing about five liters of filtered seawater. The jars rested on a heavy table placed on the concrete floor of an isolated house built directly on the rock of Agar's Island. The animals were observed continuously for an hour, and the time of each spontaneous contraction was noted. Figure 13 gives a graphic account of two such animals. It is very evident that there is a rhythmic occurrence of the spontaneous movements.

By means of the apparatus which has been previously described (fig. 7), kymograph records were made of this rhythmicity. Animals were allowed to register their activity at times of the day and night when Agar's Island was deserted except for the presence of two people in a house more than a hundred yards from the laboratory. The curves in figures 14 and 15 show the movements of two animals which are entirely typical of all the others. There can, therefore, be no doubt of the rhythmic character of the spontaneous contractions exhibited by *Ascidia*.

The rhythmicity of the movements possesses a peculiarity which resemble the refractory properties of the vertebrate heart (Woodworth, '02). It is well known that immediately after a contraction of the ventricle, it fails to respond to stimulation. After this refractory period, an external stimulus will cause a pulsation even before the expected rhythmic contraction is due. Similarly in *Ascidia* a stimulus which is so slight that it causes merely a siphon rim movement will, when applied regularly at intervals of a minute, call forth complete body movements at approximately the periods when they are expected to occur rhythmically. The following record is chosen as an example because the spontaneous contractions of this animal have already been recorded (figs. 13 and 14).

Exp. VI.27.3. Animal in a nine liter battery jar. Stimulated every minute by the impact against the jar of a pendulum bob swinging from a distance of five centimeters.

| | | | |
|-------|------------------------|---|---|
| 12:21 | Siphon rim movement | | |
| 22 | " | " | " |
| 23 | " | " | " |
| 24 | " | " | " |
| 25 | Complete body movement | | |
| 26 | Siphon rim movement | | |
| 27 | " | " | " |
| 28 | " | " | " |
| 29 | Complete body movement | | |
| 30 | Siphon rim movement | | |
| 31 | " | " | " |
| 32 | " | " | " |
| 33 | Complete body movement | | |

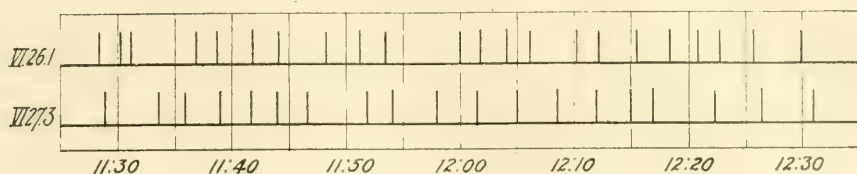


Fig. 13 Graphic record of occurrence of spontaneous movements.

Comparison with the other records (figs. 13 and 14) shows that the complete body movements occur at intervals similar to their rhythmic appearance spontaneously. The significance of this type of experiment I believe to be as follows. After the

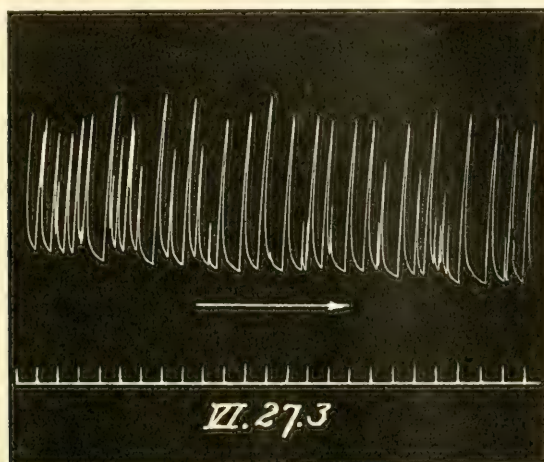


Fig. 14 Records of rhythmic, spontaneous movements. The base line marks five minute intervals.

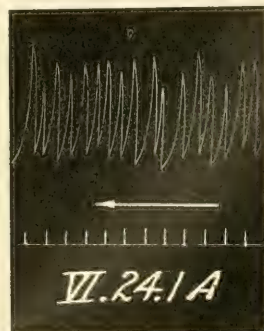
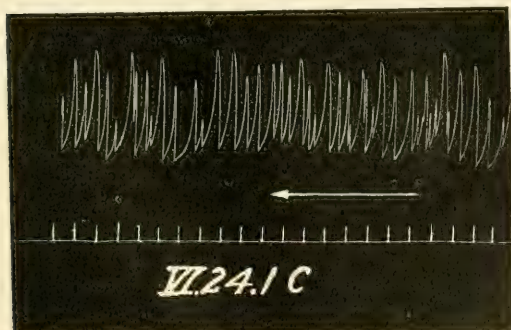


Fig. 15. Records of rhythmic, spontaneous movements. The base line marks five minute intervals.

performance of a rhythmic contraction, *Ascidia* passes through a period during which a certain strength of stimulus fails to call forth a movement of the body. However, as this refractory period passes by and the time approaches for the culmination of the next rhythmic movement, this same sub-liminal stimulus will call forth not only the usual siphon contraction, but also the body contraction in advance of its scheduled time.

The function of these rhythmic movements is by no means clear. Their accomplishment of a partial discharge of the water in the branchial sac would be intelligible if something else besides the water were also expelled. *Ascidia*, however, possesses an effective mechanism for avoiding just such a necessity. The tentacles in the oral siphon screen out all but the smallest particles which come in with the water current. Everything that passes beyond them is incorporated into the food cord in the branchial sac. The particles which are large enough to touch the tentacles, set off a reaction that drives the water and the particles out of the cavity of the siphon. Substances in suspension either get into the branchial sac and stay there, or they are forced out at once. Therefore, the function commonly attributed to rhythmic movements in other animals (Redfield, '17) cannot apply in the case of *Ascidia atra*.

To call the movements a respiratory rhythm would also fail to explain their existence. *Ascidia* has a highly efficient respiratory mechanism which moves large quantities of seawater. The renewal of the contents of the branchial sac by the rhythmic discharges would be of no significance compared to the continuous stream of water produced by the cilia. The relative infrequency of the discharge would also argue against a respiratory rhythm.

The spontaneous movements in *Ascidia* are not an isolated instance. I have observed them in a colonial species, *Ecteinascidia turbinata*, the individuals of which are about two centimeters long and quite light in weight. When the animals are attached, the effect of the body contraction is solely to discharge the water. If, however, an individual is removed from its attachment and placed in a large jar of seawater, it jerks itself along the bottom in a manner that vividly recalls the behavior of *Salpa*

under similar conditions. The frequency with which *Salpa* pulsates is several hundred times as great as those with which *Ascidia* and *Ecteinascidia* perform their spontaneous contractions.

These facts as well as the apparent lack of function of the rhythmic movements have led me to suggest that perhaps the rhythm is the degenerate remains of a once vigorous activity. The ascidians are generally supposed to have originated from the free swimming appendicularians. These possess no tentacles, and most probably the earliest ascidians did not possess them either. It is, therefore, entirely intelligible that the rhythmic discharge of the water from the branchial sac of these ancestral ascidians was of considerable value as a cleansing process. Moreover, the salpas are derived from the early ascidians. With their specialization for a pelagic existence, the rhythmic movements were developed into a mechanism for respiration, feeding and locomotion. The stem line of ascidians, however, soon developed tentacles, and the rhythmic discharge of the branchial sac contents, therefore, decreased in importance. The frequency with which it occurred probably also decreased. On the basis of this hypothesis, there exist at present two divergent lines of development of the spontaneous movements. One of these constitutes the salpas, whose frequency of contraction is several hundred times greater than that of the ascidians, which constitute the other line.

V. SUMMARY

1. The blue-black color of *Ascidia* is due to the presence of spherical pigment granules, which are the metamorphosed remains of the green blood cells. Before becoming imbedded in the test, the green cells turn blue, and may be found as such in the blood stream.

2. *Ascidia* is capable of regenerating its test. The process of regeneration and the normal sloughing of the test show that there is a continuous secretion of material on to the inner face of the test.

3. A species of amphipod lives commensally in the branchial sac of *Ascidia*.

4. *Ascidia* maintains a seawater current of large volume and low pressure. The volume of the water moved per unit weight decreases as the size of the animal increases. The difference in intensity and direction between the incoming and outgoing currents enables *Ascidia* to secure a continuous supply of fresh seawater independent of the movements of the sea.

5. Food collection is accomplished in the branchial sac with the aid of the papillae and the mucus secreted by the endostyle. The food particles are transported in a mass across the face of the branchial sac, dorsally and posteriorly into the oesophagus.

6. *Ascidia* cannot survive solely on the dissolved organic contents of the seawater. It must be furnished the suspended contents as well.

7. The species is capable of three kinds of movement: the siphon rims can close and open, the body can contract along the dorso-ventral axis, and the entire animal can bend toward its base of attachment. The movements are accomplished by several sets of smooth muscle, which depend for their proper action on the function of the test as an exoskeleton.

8. *Ascidia* performs rhythmic movements to discharge the contents of the branchial sac. No function can be ascribed to this rhythmic occurrence, and a suggestion is made that it may represent the degenerate remains of an activity homologous with the rhythmic pulsation of the salpas.

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THE PHYSIOLOGY OF ASCIDIA ATRA LESUEUR¹

II. SENSORY PHYSIOLOGY

SELIG HECHT

TWO FIGURES

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I. THE RESPONSES OF ASCIDIA AND THEIR NERVOUS RELATIONS

The assumption of a sessile mode of life involves a sacrifice in the number and kinds of responses of which an animal is capable. The comparatively few reactions exhibited by the sessile tunicates are undoubtedly accountable for the almost complete absence of our knowledge of their sensory physiology. The common European ascidian, *Ciona intestinalis*, is the only one in which anything is known of the behavior under stimulation.

¹ Contributions from the Bermuda Biological Station for Research, No. 79, and contributions from the Zoölogical Laboratory of the Museum of Comparative Zoölogy at Harvard College, No. 304.

Even here, however, the data are meager and scattered, and consist largely of incidental observations. This lack of knowledge and the abundant presence of *Ascidia atra* at Bermuda served as incentives for the following investigation of the sensory reactions of this species.

1. *Description of reactions*

Jordan ('07) on the basis of his observations has called the ascidian *Ciona*, an animal poor in reflexes (reflexarmes Tier). Without subscribing to any of his theoretical generalizations, which Baglioni ('13) has justly criticized, I have no hesitation in similarly describing the behavior of *Ascidia atra*. Tests with a variety of conditions of stimulation have revealed very definite activities, by means of which the animals respond to changes in the environment. The number of these activities, however, is small.

The structures and movements which are involved in their execution have already been described (Hecht, '17). It remains to explain their relation to one another and to the source of stimulation. The presence of the open siphons and of the water current makes it possible for *Ascidia* to receive indications of changes of the environment not only on its exterior, but also on its interior surfaces. This distinction is of fundamental importance, because the place of reception of the stimulus determines the kind of movement which the animal executes. As a result there are manifested two qualitatively distinct groups of reactions. Each of the groups consists of three responses, which involve the use of different combinations of muscles.

The group of *direct responses* depends for its origin on a source of stimulation which affects the *external* surface of the animal. This group of responses is concerned mainly with mechanical stimuli. Although the three reactions included under this head result from different intensities of the same outside disturbance, the reactions themselves involve an activity of different muscles, and not a different degree of activity of the same effectors.

1) If the test of *Ascidia* be touched very lightly, the siphon nearer the point of stimulation will contract. The extent of the

resulting closure depends on the intensity of the stimulus and on its distance from the siphon. After a short interval the siphon rim opens and the animal is normal again.

2) If, however, the stimulus has been stronger, not only does the siphon rim nearer the stimulated area close, but the other siphon rim also closes. A new set of muscles has been called into play.

This reaction is to be differentiated from the one in which both siphons are stimulated, such as when a drop of water is allowed to fall on the surface of the water in the aquarium. In this case each siphon is independently stimulated by the same disturbance. Such a reaction persists when all nervous connections between the two siphons have been cut (cf. Loeb, '92 and Magnus, '02). It is otherwise with the response which I have described. Normally, if one siphon is touched so carefully that the animal is not jarred, both siphons will close provided the proper intensity of stimulus is used. When, however, the nervous connections between the two siphons is severed, only the stimulated siphon rim contracts.

3) In response to an ordinarily vigorous mechanical stimulus, *A. atra* reacts by the employment of still an additional set of effectors, the longitudinal muscles of the body. Not only do both siphons close, but the body bends on its long axis toward the right side.

This bending toward a structurely determined side is of significance in the ecology of *Ascidia*. Most individuals of the species are attached with the body projecting at any angle, but mainly in a nearly horizontal plane. All such animals which I examined, were found with the left side of the body uppermost. Consequently, the curving toward the right side results in bringing the siphons into such a position, that a disturbing body on the outside will roll off, and one on the inside will fall out.

In the previous work on ascidians the reaction which involves the bending of the body has been the only one which has received any adequate attention. It has been generally regarded as the only reflex of which this group of animals is capable, and there-

fore called 'the reflex' (Loeb, '02). Jordan ('07) has more appropriately called it the protective reflex (Schutzreflex).

The reactions which are comprised in this direct group show individual variations depending upon the intensity of the stimulus which sets them off. They can, however, be very definitely separated from one another when the animal is observed with any degree of care, or when graphic methods are employed. (See for example, figure 8 of the first paper of this series: Hecht, '17.)

All these reactions are to be kept apart from those which are in the group of *crossed responses*. The stimuli which result in the reactions of this second type are all localized on the *interior* surfaces of the siphons, of the atrial cavity, and of the branchial sac. They include changes in the environment not only of a mechanical nature, but of a thermal, photic, and chemical kind as well.

Although they may be produced by the same kind of stimulus varying in intensity, the three reactions included in this group are, nevertheless, the result of different combinations of effectors. As in the case of the direct reflexes, they must, therefore, be sharply distinguished one from another. To make this clearer, I may refer to the behavior of a human being suddenly exposed to a bright light. The person will reflexly close his eyes. If, however, the light be made excessively bright, he will not only close his eyes but also place his hand over them. The nature of the stimulus is the same, but the greater intensity of the stimulus brings forward a new activity superimposed upon the simple eye closure. The same is true of the following reactions of *Ascidia*.

1) An exceedingly delicate stimulus on the inside of one siphon results in a closure of the other siphon. The stimulated siphon remains wide open, while the sphincter of the other siphon is called into play. This kind of response can be secured only under very carefully controlled conditions. The animal must not be jarred and the stimulus must be a delicate one. It is best to use large animals because they are not as sensitive as the smaller ones.

2) An increase in the intensity of the stimulus produces a reaction which does more than merely stop the water current; in

addition, it brings about a discharge of the water present in the branchial sac. The stimulated siphon remains open, the other siphon closes tightly, and the animal contracts vigorously along its dorso-ventral axis, resulting in a sudden decrease in the capacity of the respiratory chamber. Occasionally the stimulated siphon may also contract partially so as to decrease the size of its opening. This gives the ejected water a greater momentum.

3) The last reaction of this group combines a bending of the body on its long axis with the movements of the previous response. This is the usual reaction which *A. atra* gives under ordinary conditions of stimulation of its internal surfaces.

The last two reactions probably correspond to what Jordan ('07) has described in *Ciona* as the 'Ejektionsreflex': "Closure of one siphon, rapid contraction of all muscles, other siphon (most frequently, but not always, the anal siphon) remaining open" ('07, p. 98). This description is repeated by Polimanti ('11), who, however, added nothing to it. Jordan did not study this reflex at all, but contented himself with the statement that it serves to throw out foreign bodies, and that the causes for its appearance are not clear.

In *Ascidia* there is no doubt about the nature of the stimulus which will produce any of these three crossed reactions. It is always a disturbance on the interior surfaces of the body. I have observed the same 'Ejektionsreflex' in the common *Ecteinascidia turbinata* of Bermuda under the same conditions of stimulation as in *Ascidia atra*. Jordan's statement of its function is correct; it must, however, be broadened to include not only the ejection of foreign particles, but also the response to any internal irritation, such as strong light or chemicals.

The point of special significance is the crossed behavior of the siphon rims. Stimulation of the outside of a siphon causes that siphon rim to close. Stimulation of the inside of a siphon results in that siphon remaining open while the other siphon rim contracts. This points to the presence of a complexity of innervation in ascidians of which there has previously been no suspicion.

The one factor which the six reactions of *Ascidia* possess in common is their negative character. A source of stimulation

is either excluded by the closing of the entrances to the body, or it is thrown out by a discharge of water. I have never observed any positive response to a stimulus in this species. This is not unexpected from its mode of existence. The animals are entirely dependent for their supply of energy on what is brought in by the water current, and they merely exercise a choice by rejecting anything which acts as a stimulus.

2. *Nervous relations*

Among higher animals, the tunicates are peculiar in the concentration of the entire central nervous system into a single inter-siphonal ganglion. In *Ascidia atra*, according to Hilton ('13),² this is a roughly cylindrical mass, on one side of which is to be found a rather unusual neural gland (Metcalf, '00). It gives off many more nerve trunks than are usually described for this genus of ascidians. From the oral end there arise three large nerves, which go to the region of the oral siphon. Several nerves leave the atrial end, while from the middle of the ganglion there emerge three large nerves, four smaller ones, and many minute ones. It is significant that all the nerves contain both afferent and efferent fibers (Hilton, '13, p. 116).

Practically nothing is known of the nerve endings in ascidians. The same may be said of the presence of sense cells. Hilton describes the fibers of the oral nerves as ending in the oral tentacles, but fails to state whether they form free nerve terminations or arise from sense cells. Lorleberg ('07), after prolonged investigation of the nervous system of *Styelopsis*, concludes that there is a complete lack of sense cells, but that there are undoubted free nerve terminations present.

In relation to the reactions of ascidians, one point is clear: the only demonstrated means of direct nervous communication between the siphons is by way of the ganglion. The ganglion, however, has more than the mere conducting function supposed

² This author refers to the species as *Tunica nigra*. I have it from Professor Mark that Hilton's work was done on *Ascidia atra*. Moreover, his description of the species as the "ascidian very abundant on Agar's Island" leaves no doubt as to its identity.

by Loeb ('92). Although some of the results of Fröhlich ('03) on the removal of the ganglion of *Ciona* have been questioned by later authors (Jordan '07, and Kinoshita '10), the combined work of all the investigators on *Ciona* proves that ganglion removal affects at least the threshold sensitivity, the tonus, and the rate of recovery after stimulation.

Ascidia does not remain normal in the laboratory long enough to permit of a study of the quantitative effects of ganglion removal. I had, therefore, to content myself with a determination of the qualitative results produced by the mere nervous isolation of the two siphon regions from each other. This was accomplished by means of a rapid incision into the test and mantle so directed as to result in the severing of the nervous mass into two parts. The animal recovered from this slight operation in a few minutes.

The behavior of individuals under such nervous conditions was very instructive. Of the group of direct reactions, the first persisted, and seemed, qualitatively at least, to be normal. The second reaction, that is, the closure of both siphons, disappeared at once. As long as the whole animal was not jarred, no amount of contraction of one siphon called forth a similar response of the other siphon. The reaction involving the body flexure depends mainly on the bending of the oral siphon. Therefore when this siphon was stimulated the bending occurred, but the atrial siphon still remained unaffected.

The essential element of the group of crossed responses is the closure of the siphon which is not stimulated. This element completely disappears after the operation. Stimulation of the inside of the oral siphon, frequently even when strong enough to involve the dorso-ventral contraction and the body bending, fails to affect the atrial siphon, and only causes a partial contraction of the oral one. Irritation of the inside of the atrial siphon brings about no change at all in the oral.

These experiments leave no doubt of the ability of each portion of the animal to perform its part of a reaction even though it is isolated nervously from the rest. The reaction of the animal as a whole, however, depends on its nervous system being intact.

II. MECHANICAL STIMULATION

1. *Touch*

Ascidia atra is an animal that under normal conditions is stimulated preëminently by mechanical means. This is the only variety of stimulus which is capable of calling forth all the possible responses of the species. The selection of its food—if mere exclusion may be called selection—is made on the basis of size, and rejection depends on the mechanical stimulation by the larger particles. The remarkable sensitivity to touch was known to even the oldest zoölogists who concerned themselves with the study of the large monascidians. Its very delicacy in *Ascidia atra* was a stumbling block to locating precisely the sensitive regions.

The presence of a heavy cellulose test would suggest an insensitivity of the exterior to any stimulation. Yet, even a gentle touch on the surface of the body results in a reaction of the direct type. Careful experimentation has convinced me that this is not due mainly to an irritability of the test to mechanical stimulation. An individual normally attached to a rock, and removed to the laboratory with its attachment intact, serves best for this type of experimentation. Moreover, if the substrate be securely clamped in the aquarium, the accidental jarring of the animal may be almost completely eliminated. Under these conditions a gentle touch with a glass rod on the test surface leaves the animal undisturbed. A coarser application at once stimulates.

I am not prepared to deny the presence of touch receptors on the surface of the test. But I am convinced that most of the results of mechanical stimulation of the test are not due to sense organs within it, but to the passage of the stimulus through the elastic material to the more sensitive region of the siphon rim. In favor of this view is the lack of any demonstrable nerve connection between the test and the tissue underneath it. Moreover, sources of stimulation, such as light, heat, and chemicals, which cannot easily be transmitted along the test substance, fail to be effective when applied to the outside of the test; whereas in all other regions, they are just as effective as touch.

The reaction to mechanical stimulation of the test is not due to an irritation of the underlying mantle tissue. Individuals whose tests have been removed from a portion of the body show that the mantle is insensitive to touch. It is interesting to explore the sensitivity of such an animal. Even vigorous poking of the mantle (the animal must be rigidly clamped, of course) is followed by no effect. One may approach to within one millimeter of the cut test and produce no stimulation. But once the test is touched, the animal immediately gives its characteristic response. An animal wholly denuded of its test is insensitive to touch on the outside except near the rims of the siphons.

In the normal animal as one approaches the region of the siphons the sensitivity to mechanical stimulation rises rapidly, and at the rim of the siphons the irritability is very great. The rim of the oral siphon is usually divided into eight lobes, and the atrial into six lobes. These thin lobes are the most sensitive portions of the outside of the body. By using large animals that have been a few days in the laboratory, and stimulating the individual lobes with a fine glass rod, I have secured local contractions of the portion of the rim contiguous to the stimulated lobe. The folds between the lobes are only slightly less sensitive than the lobes themselves.

On the inside of the siphons below the lobes, a similar degree of sensitivity exists. Inside the atrial siphon the irritability is greatest near the rim, but the entire atrial cavity is also sensitive to touch. Within the oral siphon the surface is extremely sensitive, and remains so as far down as the ring of oral tentacles. Beyond this the sensitivity falls off rapidly.

Of the surfaces which produce the group of crossed reactions, the tentacles are probably the most sensitive. The prettiest automatic response of *Ascidia* results from their stimulation. By illuminating the inside of the oral siphon it is possible to touch a single tentacle with a fine glass rod. If a delicate stimulus be applied carefully, it is most interesting to see the atrial rim close quietly while the oral siphon remains undisturbed. If the stimulus is more intense the 'Ejektionsreflex' is produced. When a

small particle of sand is dropped carefully upon the tentacles, the slight back pressure produced by the closing of the atrial rim at once squirts the particle out of the oral siphon.

In view of the certainty and ease with which these reactions may be demonstrated, not only in *A. atra*, but also in *Ecteinasidia turbinata* and in another unidentified species, it is difficult to understand why some authors have reported that the tentacles are practically insensitive to mechanical stimulation. Thus Roule ('84, p. 37), who studied *Phallusia*, and Lacaze-Duthiers et Delage ('99), who observed *Cynthia*, state that no noteworthy reaction occurs when the tentacles are touched in this way. This is all the more strange because it is precisely here that Seeliger (1893-'11, p. 323) has found most of the bristle cells to which he rather doubtfully ascribed the rôle of touch receptors.

The perception of mechanical irritation by the internal surface of the atrial siphon is of significance in the daily routine of the species. A decidedly sensitive area is at the bottom of the atrial cavity near the anus. The feces are discharged into this cavity. Here they furnish the mechanical stimulus for a reflex of the crossed type: the oral siphon closes and the body contracts, squirting the water and the feces out through the atrial siphon.

To one unacquainted with the presence of the group of crossed reflexes, the defecation of *Ascidia* seems almost a conscious procedure. It 'tries' to force out the feces, and if a piece becomes caught in the siphon rim or in the atrial cavity, it 'tries' again to dislodge it by means of the ejection reflex, until finally it succeeds. The whole process can, however, be called forth by placing a glass bead or a pebble in the atrial cavity, or by repeatedly stimulating it with a glass rod.

2. *Vibration*

The extreme sensitivity of *Ascidia* to mechanical stimulation is manifested in its ability to respond to vibrations (compare Marage, '05). *Ascidia* lives in shallow water, and if the rocks within two or three meters of an individual are stamped upon with even a modicum of vigor, it closes its siphons. A rapid motion in the water within the same range also serves as a

stimulus. In the laboratory an animal in a battery jar on a table will react when a person walks vigorously across the floor. A sharp tap on the glass walls of the aquarium results in an extreme reaction of the animal as a whole.

It is hardly possible to exaggerate the sensitivity of *Ascidia* to this kind of stimulus. Its very delicacy was at first a source of interference in the study of the sensory physiology of the species. The plan was, therefore, adopted of working in an isolated house the concrete floor of which rested on the bed rock of the laboratory island.³ Only in this way was it possible to avoid the constant unintentional stimulation of the animal under investigation.

To determine the special region concerned with the reception of the vibrational stimuli, I had first to discover along which medium the stimulus was carried to the animal. There could be no doubt at all about the existence of a water transmission. A drop of water allowed to fall on the surface of the water in the aquarium sets up a series of waves which are very effective in stimulating the animal. Moreover, it was easy to cause a reaction by dropping a solid into the water near an individual in its natural surroundings. When an animal, submerged in the water of a large battery jar, is suspended from the ceiling in such a way as to prevent its touching any portion of the jar, the stimulus from a tap on the side of the jar will be transmitted to the animal by way of the water only. Under such conditions, a sharp tap called forth an immediate reaction. Therefore the vibration in the water serves as a stimulus.

The question whether *Ascidia* is stimulated by vibrations transmitted through its solid base of attachment was answered in the affirmative by an experiment like the following. An animal was placed upright on the bottom of an aquarium. Over it was then inverted a battery jar which was a centimeter or so greater in diameter than the width of the animal. The jar rested on the glass bottom of the aquarium, and sufficient cotton was put under its rim to remove any direct transmission of vibrations to the animal by means of the water in the aquarium.

³ To those familiar with Agar's Island:—the cook-house.

Under these arrangements, a tap on the glass aquarium still resulted in a reaction. Therefore, there seems to be a reception of the stimulus by way of a solid medium too.

It was possible to determine the relative effectiveness of stimuli transmitted by the two pathways, by measuring the quantity of energy which must be expended to produce the same reaction in the two cases. A pendulum bob weighing nearly 75 grams was suspended from a string about 150 centimeters long, in such a position that when at rest it hung 5 millimeters from the wall of the aquarium. The string was made long so that the velocity of the pendulum bob would be practically the same when it swung from any distance within about 35 centimeters of the position of rest. In this way the amount of energy which the impact of the bob delivered against the side of the jar varied with the square of the distance from which it was released. In one experiment, to quote a typical example, a swing of 10 centimeters was enough to produce a complete siphon closure when the vibrations were transmitted through the water only; whereas it required a swing of 30 centimeters to produce the same reaction when only the solid transmission occurred. The vibration through the water was therefore nine times as effective as the vibration through the glass.

If, now, we keep in mind that *Ascidia* is very sensitive to even slight vibrations, it seems clear that it is their occurrence in the water which furnishes the stimulus under normal conditions. Stimulation through the solid base of attachment is most likely a wholly secondary phenomenon. Hence, in looking for the structure where the reception of the vibration occurred, I had to consider only those parts fully exposed to the water.

No statocysts have been described in adult ascidians; such sense organs occur in the larva, but disappear during the metamorphosis (Herdman, '04). There is a space between the test and the left side of the body, which usually contains some fluid. I, therefore, tested the possibility that there might be some equilibrium relation between the test and the body which would be influenced by the vibration. The animals were suspended in all sorts of positions calculated to disturb this relation, without,

however, affecting the sensitivity in the slightest. The equilibrium idea was therefore incorrect.

The ring of very sensitive oral tentacles is in direct communication with the surrounding water. The tentacles are thin-walled protrusions of the branchial sac, and remain in a more or less rigid extension due to the pressure of the blood which circulates freely within them. It might be imagined that there existed an arrangement similar to the hair-crowns of mammals, which would make them receptors for the vibrations in the water. Cutting away all the tentacles was a simple matter, except that it involved the use of an anesthetic (chloral hydrated). Recovery, however, was very rapid and in an hour the animals were normal again. After this procedure, they were as sensitive to vibration as before the operation. Moreover, as will be recalled, touching of the individual tentacles produces a crossed reaction; this is not the kind of response which the animal gives to vibration. Therefore the tentacles are not concerned with the reception of vibratory stimulation.

The only other regions acutely sensitive to touch are the lobes of the siphon rims. In the expanded animal these thin flaps protrude prominently into the surrounding water. If a specimen is suspended with the siphons hanging down, the water in the aquarium may be withdrawn so as to leave only the rim of the oral siphon immersed in the water. When this is done carefully the animal does not close its siphons. Under these conditions the oral siphon will react when the aquarium is tapped. Removal of the lobes from the rims of both siphons does away completely with the sensitivity of the animal to vibrational stimuli. The conclusion, therefore, seems inevitable that the thin, projecting lobes of the siphon rims contain the receptors for this kind of environmental disturbance.

3. Repeated stimulation

The continued application of vibratory stimuli at regular intervals results in a cessation of response on the part of the animal. Whether this failure to react is the result of the fatigue of the musculature, or of the sense organs, or whether it is an

act of adaptation, it is not possible to state with any degree of confidence on the basis of the mere observation of the animal. It seemed therefore advisable to record the amplitude of response during the period of repeated stimulation. In this way it was hoped to gain at least a clearer presentation of the problem.

Accordingly, an apparatus was arranged by means of which the amplitude of any reaction would be recorded on the slowly moving drum of a kymograph. The animal was stimulated by the vibrations produced in the water by the impact against the glass aquarium of the pendulum bob previously used. It was found more satisfactory to apply a stimulus just as the animal had recovered from the effects of the preceding stimulus. This, in fact, amounted practically to a stimulation at regular intervals of about one minute.

In figure 1, is shown the record⁴ of a series of contractions produced by the impact of the bob swinging from a distance of 30 cm. The animal failed to contract after a half hour of regular application of the stimulus. If the shape of the record be compared with the fatigue curve of a voluntary muscle within the organism (Howell, '12, p. 48), their similarities with regard to the presence of a fatigue level and of a rapid final decrease in amplitude will hardly be doubted.

The presence of an appearance like that of the fatigue level of vertebrate muscle may be shown better when the stimulation is not so intense. For instance, figure 2 records the results of a regular stimulation by the bob swinging from a distance of only 20 cms. The animal responded for an hour, the arrow at point *a* indicating when stimuli applied at intervals of 15 seconds failed to call forth a reaction. An inspection of the record shows that for about three-quarters of an hour the amplitude of response to each stimulus was practically the same. At the close of the period the amplitude decreased suddenly and the animal ceased to respond. These are precisely the effects exhibited by a muscle under similar conditions.

⁴ For the apparatus by means of which these records were made, see figure 7 in the first paper of this series (Hecht, '17).

If these phenomena are to be explained in terms of the animal's adaptation to the repeated stimulus, as maintained by Kinoshita ('11) for *Ciona* and *Styela* (Parker, '17, p. 221), it follows that the continued rhythmic application of the stimulus should cease to be effective after the animal has once stopped reacting to it, that is, once it has adapted itself. In *Ascidia* this is not the case. In the curve in figure 2, for instance, the break after the point marked *a* means that the animal failed to respond

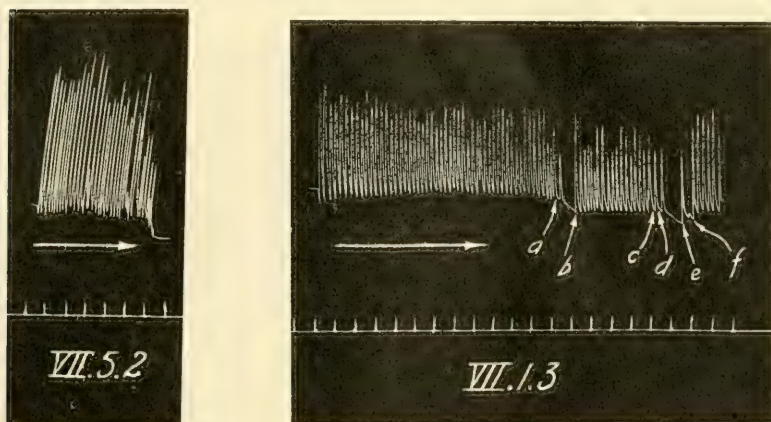


Fig. 1 Record of response to intense, rhythmic, mechanical stimulation, resulting in a rapid exhaustion. The base line marks five-minute intervals.

Fig. 2 Record of response to rhythmic, mechanical stimulation, less intense than in figure 1. For further explanation see test. The base line marks five-minute intervals.

though stimuli were applied at the rate of one every 15 seconds. This continued for three minutes when suddenly it again began to react and continued to do so for twenty minutes. At the point marked *b* the animal failed to contract to one impact but responded to the next (*c*). It then ceased responding for four more stimulations. The fifth produced a contraction (*d*) but several more after that failed to call forth a reaction. A process of adaptation can, therefore, hardly be said to be responsible for the cessation of the reaction.

The fatigue of the muscles which cause the contraction is also not at the bottom of the lack of response. When an animal failed completely to respond to vibrations, a different stimulus, such as the application of heat or chemicals, at once resulted in a reaction. Also, as Kinoshita ('11) showed for *Ciona*, the cessation of response when a certain spot was regularly stimulated is concerned with that spot only, because immediate irritation of a different locality at once called forth a reaction. Therefore the phenomena are not concerned with the exhaustion of the muscle layer.

All these occurrences in *Ascidia* are best explained on the assumption that the cessation of reaction is due to the fatigue of the receptor. This accounts for the localization of the effect, and for the response to a different quality of stimulation. Moreover, it makes a record like that of figure 2 intelligible. In the first interval during which the animal did not respond, the sense organ recovered to the extent of being efficient for quite a while. But as the process of fatigue was continued, recovery occurred only to the extent of enabling the sense organ to receive a single stimulus now and then.

The further history of an animal in such a condition is instructive. If it be allowed to remain undisturbed for a few minutes, almost complete recovery may occur. For example, the animal whose record is given in figure 2 was not stimulated for five minutes after the point *d*. During this time there occurred a spontaneous contraction (*e*) the nature of which has already been discussed (Hecht '17).⁵ At the point *f* stimulation was again begun. It will be seen that the amplitudes of the contractions are comparable to those produced at the beginning of the record. After about ten minutes the experiment was discontinued because of the sheer exhaustion of the observer.

These experiments leave no doubt that fatigue is a factor of major importance in the effects of repeated stimulation. When

⁵ The appearance of the usual rhythmic contractions in fatigue experiments shows also that we are not dealing here with the exhaustion of the muscles, but rather with the fatigue of the sense organs which furnish the Anlass for the muscular contraction.

the same quality and intensity of stimulus is applied at regular intervals to a given region, *Ascidia* ceases to react, most probably because the irritated sense organs have become so changed that they are incapable of receiving the stimulus.

III. LIGHT

The dark-blue pigment in the test of *Ascidia atra* is undoubtedly an efficient light screen. Not only is the test well supplied with this pigment, but the inner surfaces of the siphons are pigmented well into the branchial sac and atrial cavity. The outside faces of the oral tentacles are also pigmented. Individuals found in exposed situations possessed a tough test, which was so colored as to justify unreservedly the name of the species. Specimens collected from under stones, and hence screened from the glare of the sun and the action of the waves, had a partially translucent, softer test not heavily supplied with pigment. These facts would seem to indicate a sensitivity of this species to light.

Many ascidians are described as possessing red or orange pigment spots near the rims of the siphons (Seeliger, '93-'11, p. 319). *A. atra* has eight of these 'ocelli' on the oral siphon and six on the atrial siphon; they are situated in the regions of the less pigmented folds of the test between the lobes of the siphon rims. In this species their color is due to an aggregation of pigmented cells which resemble, if indeed they are not identical with, the orange blood corpuscles found in the branchial sac. Although no evidence has ever been adduced for it, the idea has been prevalent that these ocelli are photic sense organs.

A sensitivity of the body surface to photic stimulation has been ascribed to *Ciona* by Kinoshita ('10) on the basis of its reactions when carried from a dark room into diffuse light. It is difficult, however, to accept this interpretation with much confidence, because the author records no precautions to avoid the jarring of this highly sensitive animal (Fröhlich, '03) during the transfer. However, Nagel ('96) has also stated that "*Ciona* reacts powerfully to sudden illumination."

Ascidia is insensitive to light of ordinary intensities. Animals kept in the dark and suddenly illuminated by an electric light,

or by diffuse light, or even by direct sunlight, remained undisturbed. Rays from an electric bulb, concentrated to a point and focussed on the individual ocelli failed to call forth any response from the animal. By means of a large lens whose focal length was 30 cm., direct sunlight was focussed upon each ocellus in turn by way of the outside and the inside of the siphon. In no case did this result in a reaction. I must, therefore, conclude that the so-called ocelli of *Ascidia atra* do not contain receptors for photic stimuli.

Nevertheless, this species is sensitive to light of great intensity. If direct sunlight is allowed to pass through 36 cm. of seawater and is then concentrated by means of a lens, there is no evidence of the heat that is associated with it. Such a beam of light produced no sensation of heat when focussed on a finger under water, nor a rise in temperature when directed upon the bulb of a thermometer. As was to be expected, when this light was concentrated on the ocelli, the animal again failed to respond. It also called forth no response when focussed on any portion of the test. If the beam was, however, directed into the oral siphon in the general region of the tentacles, about one or one and a half centimeters below the ocelli, the animal responded at once. The reaction was of the crossed kind in which the oral siphon closed only partially, the atrial completely, and the body contracted vigorously, forcing the water from the branchial sac out through the oral siphon.

When *Ascidia* was kept in broad sunlight, even if the rays entered the oral siphon, the animal remained undisturbed. If the sunlight was suddenly intercepted by means of an opaque screen, no reaction was observed. The same results were obtained when the pigment spots of the siphons were illuminated for a time and then suddenly darkened. These facts indicate that *Ascidia* is insensitive to a sudden diminution of the intensity of the light to which it is exposed under the normal conditions of its existence.

During the course of the summer, I repeated all of these experiments with light on many occasions. The results as I have described them were identical every time. Therefore, although

Ascidia is sensitive to very intense light, it can hardly be called photosensitive in the ordinary use of the term. Moreover, like the anterior pigment spot of *Amphioxus*—the 'eye-spot,' which Parker ('08) has shown to be insensitive to light—the inter-lobal pigment spots of *Ascidia*—the so-called 'ocelli'—are also not photoreceptors.

IV. TEMPERATURE

1. *Thermosensitivity*

The reactions to temperature changes are practically an unknown factor in the physiology of ascidians. The effect of different temperatures on the reactions of *Ciona*, which Jordan ('07) has investigated, is a matter entirely different from the reactions produced by the sudden exposure of the body to different temperatures. The latter effects were studied by Kinoshita ('10) on *Ciona* on the assumption that the siphon rims were thermosensitive.

In *Ascidia* the siphon rims are not sensitive to heat. As has been described, direct sunlight concentrated on the outside and inside of the rims failed to produce any response. Such a beam of light when focussed on a piece of paper caused it to burn in very short order; when directed on the bulb of a thermometer in the position of the animal, it made the mercury thread fairly leap upward. The animals, however, failed to respond. Therefore, if *Ascidia* is sensitive to temperature changes, the irritability is not resident in the siphon rims.

When *Ascidia* is picked up carefully, it merely closes its siphons. If it is at once replaced into seawater, the time required for the siphons to open again is practically constant for each individual. The record of animal VII.18.1 will serve as an example. It was picked out of the water and replaced, and the time required for opening was taken with a stop watch. The number of seconds for four trials are given in table 1.

Assuming that the animal is thermosensitive, and that the sense organs are located on the test, we might expect that transferring the animal into seawater at a higher or a lower tempera-

TABLE 1
Opening times for replaced animal

| TIME | SECONDS |
|--------------|---------|
| 10: 10 | 12.5 |
| 10: 19 | 14.8 |
| 10: 30 | 14.3 |
| 10: 42 | 14.4 |
| Average..... | 14.0 |

ture would result in its remaining closed, or in its opening time being prolonged. Neither expectation was realized. Instead, the siphons opened in the same time as in seawater at room temperature. The above animal, when placed in seawater at 35.5°C., opened in 14.2 seconds; and in water at 42.0° it opened in 13.9 seconds. Animal VII.18.2, experimented with at the same time as VII.18.1 gave the following similar results (table 2).

TABLE 2
Opening times of replaced animal at different temperatures

| TIME | TEMPERATURE | OPENING TIME |
|--------|-------------|----------------|
| | | <i>seconds</i> |
| 10: 13 | 26.7° | 7.5 |
| 10: 25 | 26.7° | 10.5 |
| 10: 35 | 26.7° | 9.0 |
| 10: 50 | 26.7° | 9.0 |
| 11: 10 | 36.0° | 8.5 |
| 11: 30 | 40.0° | 7.8 |

The further history of the animals in this experiment, however, is significant. In the seawater at the higher temperatures the siphons opened slightly after the usual interval, but at once closed with a jerk. After a few seconds the siphons again opened only to be shut immediately, and accompanied, as before, with a partial ejection reflex.

The conclusions from these results are evidently that *A. atra* is sensitive to a temperature change, that the receptors are located within the siphons, and that the test and siphon rims are insensitive to this quality of stimulation.

2. *Range of sensitivity*

The quantitative aspects of this sensitivity were rather baffling. The use of a tube in which warm or cold water circulated, as described by Kinoshita ('10), was a complete failure because of the considerable mechanical stimulation accompanying its application to the tissues. Parker ('08) in his study of the sensory reactions of *Amphioxus* found a similar method to be ineffective and for the same reason.

The process of discharging seawater at different temperatures from a pipet also proved useless because of the extreme sensitivity of *Ascidia* to the current which was set up. To avoid this, I attempted to fatigue an individual until it no longer responded to such a current of seawater at room temperature, and then discharged seawater at different temperatures near the oral siphon. The results obtained in this way showed that the animals were thermosensitive. The method, however, proved too uncertain for quantitative measurements, tending to fatigue the experimenter as well as the animal.

An attempt in still another direction was the following procedure. An animal was fixed to the short arm of an L-shaped rod, the other arm of which was attached to a toy four-wheeled cart. This cart moved on a smooth platform parallel to a trough in which the animal was submerged. The cart ran so smoothly that even so sensitive an animal as *Ascidia* remained open and expanded while it was moved the entire length of the 30-centimeter trough. In this way *Ascidia* was converted from a sessile into a moving animal. A temperature gradient was then arranged in the trough and the animal moved into the different regions. This method, too, demonstrated the thermosensitivity of the species, because the animals gave an ejection reflex when brought into a region of higher temperature. It failed, however, to give satisfactory quantitative results.

Finally I resorted to a modification of the pipet method by means of which its objectionable feature was eliminated. The tip of the pipet was covered with a layer of fine muslin, which was securely tied as near the tip as possible. The meshes of the

muslin broke the force of the current of water which flowed from the tip of the pipet by dividing it into many minute streams. The liquid still came out in an apparently coherent mass, but its velocity and force were much reduced. One cubic centimeter of ordinary seawater from such a muffled pipet directed into the oral siphon left the animals undisturbed.

The results obtained by the use of this method were gratifyingly uniform. At the normal summer temperature of 26.0° to 27.0°C. the discharge of 1 cc. of seawater at 32.0° within five millimeters of the oral siphon produced a reaction in most cases. Seawater at 35° and at higher temperatures acted as undoubted thermal stimuli. The reactions were always of the crossed variety, which has already been described. For temperatures below that of the room, the reactions were analogous. Seawater at 20.0° very frequently acted as a stimulus. Temperatures below 20.0° were practically invariable in their stimulating effects.

From these facts it is clear that over a range of 12 degrees *A. atra* is insensitive to temperature changes. Undoubtedly this recorded range is greater than the actual one, because of the dilution and consequent change of temperature of the stream of warm or cold water. A rough approximation of the temperatures to which the animal was actually exposed is furnished by substituting a small thermometer in its place and directing the water on the bulb. The temperatures recorded by the thermometer in one such experiment are given in the following table (table 3). It must, however, be remembered that the case of a sense organ and of a thermometer are only roughly comparable, because of the much larger volume of substance which has to be heated in the thermometer bulb. The actual temperature to

TABLE 3

| TEMPERATURE IN DISH | TEMPERATURE IN PIPET | THERMOMETER READING |
|---------------------|----------------------|---------------------|
| 27.8° | 41.0° | 30.2° |
| 28.0° | 46.0° | 31.1° |
| 28.0° | 50.0° | 32.0° |
| 28.0° | 54.0° | 32.5° |
| 28.0° | 67.0° | 35.0° |
| 28.0° | 76.0° | 37.0° |

which the sense organ was subjected lies between the recorded temperature and the temperature of the water in the pipet, probably nearer the former than the latter.

The test and siphon rims have already been shown not to contain the receptors for temperature changes. Insensitivity to these stimuli extends for a few millimeters into the siphons. Below this, the interior of the oral siphon is sensitive to heat and 'cold.' Although the oral tentacles are probably concerned they are not the only regions which receive the stimulation. When the tentacles were removed, reactions to hot and cold seawater were still obtained. Therefore the wall of the siphon also contains regions sensitive to temperature changes.

V. CHEMICAL STIMULATION

1. *Presence of chemical sense*

The earliest statement of the chemical sensitivity of ascidians is to be found in Nagel's ('94a, p. 553) words that *Ciona* and "probably most sessile ascidians lack completely any chemical sense." Nagel found that the siphons of *Ciona* were retracted not only on the application of acids, but of pure water as well ('94b, p. 173). Since it was his idea that fresh water stimulated because of the mechanical disturbance set up by its discharge from a pipet, he explained the action of the acids on similar grounds.

Nagel, however, was incorrect in the interpretation of his data. This is shown by an incidental experiment of Magnus ('02, p. 485), in which the mechanical stimulation was avoided. He found that *Ciona* will respond to the presence of a crystal of sodium chloride held near the oral siphon. This sensitivity of *Ciona* to salt constitutes, as far as I am aware, the only known fact about the chemical sense of ascidians.

It was an easy matter to determine whether *Ascidia atra* was responsive to substances in solution. When a crystal of a so uble substance was held near the oral siphon, the incoming current dissolved enough of the material to stimulate the animal. If the substance was a liquid or was non-crystalline, a wad of absorbent

cotton was saturated with a concentrated solution, which was then applied in the same way as the crystal, and with the same result. By trying many substances in this way, it soon became clear that Nagel was very far from the truth when he denied a chemical sense to ascidians. *Ascidia* responded to all the acids, bases, salts, alkalkids and anesthetics with which I made preliminary tests.

It was, however, not my aim to make an exhaustive qualitative survey of this type of irritability. The effects of various substances on protoplasm have already received sufficient attention to enable one to classify them broadly into certain groups. Therefore, a representative number of substances from each of these groups were chosen for study, not so much with a view to determine their individual properties, but rather with a desire to compare quantitatively their effectiveness in producing the same response. In this way it was hoped to interpret the physiologic nature of the chemical receptors in terms of the modern concepts of the physical chemistry of cells and tissues, much as Cushney ('16) has suggested the consideration of the finer chemistry of protoplasm in terms of the recent work in pharmacology.

2. Method

Ascidia is sensitive to fresh water; therefore the materials to be tested were dissolved in seawater. Because of the low concentrations which were necessary, the osmotic pressure of the seawater was increased but slightly. Moreover, the animals are sensitive only to large changes in the osmotic pressure.

Seawater is a balanced solution. The addition of an electrolyte already present in it, tends to disrupt the balance, making the augmented substance the dominant factor in stimulation. Undoubtedly the effect of the same concentration of a substance in distilled water is greater than in such a partially balanced medium. However, the results which I obtained are so similar to those ordinarily secured in physiologic experiments by the use of single electrolytes, that I am inclined to assign little value to this source of error.

In order to avoid the mechanical stimulation of the current produced by the application of a substance, recourse was again had to the muffled pipet used in the experiments on the temperature sense. By this means the solution to be tested could be discharged into the siphons without producing any effect other than that due to the dissolved material. The muffled pipet was, therefore, used in all the experiments.

The outflow of one cubic centimeter of solution from the pipet took place at the following rate. The first quarter was discharged into the seawater in 1.2 seconds; the next quarter required 1.8 seconds; the remaining half took 5.0 seconds. The discharge of the entire cubic centimeter was accomplished therefore in 8.0 seconds. In actual practice any solution which stimulated at all, did so during the discharge of the first half cubic centimeter.

There is an inherent weakness in this method of applying a stimulus that must never be lost sight of. It is that the concentrations in the pipet do not represent the solutions which reach the sense organs, because of the diffusion of the stimulating solution into the surrounding seawater. It is difficult to form an exact estimate of the amount of this dilution. A conservative statement would be that the material from the pipet is mixed with about two or three times its volume of seawater before it reaches the inside of the siphon. The matter is simplified in *Ascidia* by the existence of a continuous current entering the oral siphon. The solution thus diffuses less than if it were not at once sucked into the branchial sac. In order to avoid still more the effects of the dilution, the tip of the pipet was kept within a centimeter of the opening of the oral siphon.

As a quantitative measure of the stimulating strength of the chemicals tested, I determined the lowest concentrations which would produce a reaction. The reaction time—that is, the time elapsing from the moment of application of the stimulus to the beginning of the reaction—cannot be used with advantage in this type of experiment. It is complicated by so many factors, such as diffusion rate and speed of penetration, that it gives little direct information. To measure the amplitude of the effects of equal concentrations of different substances requires a

nicety in the grading of a response of which *Ascidia* was not capable. The value of the liminal concentration, however, has the important advantage that it gives the quantities of material which are required to produce the same physiologic result. No other measure of stimulating strength possesses this property.

The sensitivity of individuals varied according to their size: the larger animals were less sensitive than the smaller. This is to be expected from what has been shown for the relation between certain activities and body weight; for example, the intensity of the water current and the frequency of the heart beat. An instance of this variation is the following. In one experiment I used three animals whose weight averaged nearly 30 grams. The liminal concentration for KCl was 0.075 N. In a parallel test on animals whose weight averaged 65 grams, the liminal concentration of KCl was 0.20 N.

Ascidia atra was not sufficiently plentiful for me to employ animals of one size throughout. An experiment, therefore, consisted of the testing of a group of substances on each of three similar sized individuals. The results secured in this way were uniform, and were serviceable for the desired comparisons within the groups.

The procedure was as follows. Each animal was placed, resting on its left side, in a finger bowl containing 300 cc. of seawater. Before applying a stimulus, I always waited for the culmination of a rhythmic contraction. This simulates the reaction to an irritant in so many ways, that it was deemed necessary to include it as a source of error. After each of the three individuals had been tested with 1 cc. of a solution, they were removed into bowls of fresh seawater and allowed to remain undisturbed for at least five minutes. A complete experiment involved ten or a dozen reactions of the same animals, and usually lasted about two hours.

3. Distribution of sensitivity

The test of *Ascidia* is insensitive to chemical stimulation. I have discharged concentrated HCl on various portions of the test without securing any response whatsoever. It is problematic

whether the outside margins of the siphons are sensitive. Solutions applied to the outside rim of the oral siphon are drawn inside and the resulting reaction may be due to the stimulation of the latter region. Even a minute crystal of oxalic acid applied to the outside rim caused a local mechanical disturbance. Solutions applied to the atrial rim must be much stronger to produce an effect than those applied to the oral rim. This may mean that it is not the outside rim which is stimulated, but that to affect the interior of the atrial siphon, a more concentrated solution is required in order to diffuse in against the outgoing current; or it may signify that the outside oral rim is more sensitive than the atrial. On the basis of my experiments it is not possible to decide between these alternatives.

The region of maximum chemical sensitivity is the inside of the oral siphon. A crystal of oxalic acid held on the end of a fine glass rod inside the siphon, at once produced a reaction, although, due to the incoming current, none of the dissolved acid touched anything but the interior of the siphon. There may be chemical receptors on the tentacles, but they are not of primary importance. Individuals with the tentacles removed were not qualitatively different from intact animals.

In these experiments, the values recorded are all for the sensitivity of the oral siphon. More accurate results could be obtained by using this siphon because the direction of the water current favored the passage of the stimulating solution. For the sake of comparison, however, the relative sensitivity of the two siphons to HCl was determined. One set of animals showed that the oral siphon responded vigorously to 0.002 N solution of HCl; it required, however, a 0.04 N solution of HCl to stimulate the atrial siphon. Hence the oral region is apparently twenty times as sensitive as the atrial.

4. Osmotic pressure

Ascidia did not respond when 1 cc. of seawater was discharged near its oral siphon. Seawater evaporated to twice its concentration also failed to stimulate. Seawater evaporated to four times its concentration called forth a vigorous reaction.

Closer measurements as exemplified by Exp. VIII.5 showed that seawater concentrated to be isotonic with 1.57M solution of NaCl just stimulated.

The species is also not very sensitive to diluted seawater. A mixture of seawater and fresh water in equal proportions did not result in a reaction. The animals of Exp. VIII.5 gave the first response when stimulated with a mixture of 3 cc. of seawater and 7 cc. of fresh water. This 30 per cent seawater corresponds to a 0.19 M solution of NaCl.

The range over which *Ascidia* is insensitive to changes in osmotic pressure is really not as large as it would seem from these experiments, because the concentration of the stimulating solution changes before it reaches the receptors.

To gain some information on the extent of this change, I determined the toxicity of various dilutions of seawater to *Ascidia*. Eighteen animals of nearly the same size were placed in lots of three in individual bowls containing 300 cc. of the following dilutions: 100 per cent, 90 per cent, 80 per cent, 70 per cent, 60 per cent and 50 per cent seawater. The solutions were freshly replaced three or four times daily.

In the 100 per cent seawater one individual died in less than 24 hours; the other two were still vigorous after 72 hours, when the experiment was discontinued. Exactly the same occurred in the 90 per cent seawater. The average longevity of the three individuals for each of the other dilutions are given in table 4.

From these results it is clear that 80 per cent seawater is decidedly toxic to the animals. It is generally true that an animal will be stimulated by a solution which is obviously toxic for it.

TABLE 4
Longevity of Ascidia in seawater of different dilutions

| CONCENTRATION PER CENT SEAWATER | LONGEVITY |
|---------------------------------|----------------|
| | <i>minutes</i> |
| 100 | ∞ |
| 90 | ∞ |
| 80 | 960 |
| 70 | 600 |
| 60 | 75 |
| 50 | 50 |

Therefore, a concentration of 80 per cent seawater at the sense organ would probably act as a stimulus. The measured liminal concentration was 30 per cent seawater. On the assumption that this corresponds to a concentration of 80 per cent seawater at the receptor, the 30 per cent seawater must have been mixed with at least three times its volume of seawater before it reached the sense organs. In this way we are furnished with a rough value for reducing the liminal concentrations given in the following experiments to a basis more nearly comparable with the results obtained in other physiologic processes and in the chemical stimulation of other animals.

5. Results with chemicals

Salts. The three typical salts, NaCl, KCl and NH_4Cl , were tested in order to compare the effects of the alkali cations. In table 5 are given the liminal values obtained in Exp. VII.5.

TABLE 5
Liminal concentrations of alkali salts

| SALT | CONCENTRATION |
|------------------------|---------------|
| NaCl | 0.4 N |
| KCl | 0.2 N |
| NH_4Cl | 0.3 N |

Arranged in the order of their effectiveness as stimulating agents, they show the familiar cation series:



This parallels the stimulating strengths of these cations found by Cole ('10, p. 607) for the common chemical sense in the frog.

In order to determine the effects of a group of anions, the following salts were used: KCl, KBr, KNO_3 , KI, CH_3COOK , and KSCN. The first experiments were made on small, and consequently very sensitive, animals. By this means large differences in stimulating power became evident; this is typified by Exp. VIII.3 of which the following table is a summary (table 6).

Later, in order to separate KCl, CH_3COOK and KSCN, larger, and therefore less sensitive, animals were used. Exp. VIII.4 was of this type and gave the results shown in table 7.

TABLE 6
Liminal concentrations of a series of potassium salts

| SALT | CONCENTRATION |
|----------------------|---------------|
| KCl | 0.075 N |
| KBr | 0.050 N |
| KI | 0.010 N |
| KNO ₃ | 0.15 N |
| CH ₃ COOK | 0.075 N |
| KSCN | 0.075 N |

TABLE 7

| SALT | CONCENTRATION |
|----------------------|---------------|
| KCl | 0.20 N |
| CH ₃ COOK | 0.15 N |
| KSCN | 0.10 N |

A combination of the two tables gives an anion series of stimulating power as follows:



Excepting SCN and NO₃, which are not in the usual positions, this order agrees with the familiar Hofmeister series (Höber, '14, p. 309). An absolutely complete agreement is hardly to be expected, because my tests were made in seawater. Höber ('14, p. 323) has constructed 'Uebergangsreihen,' in which he has been able to change the position of some members of this lyotropic series by altering the milieu in which the experiments were performed. Analogous to this is Cole's ('10) observation for the stimulation of the frog foot, in which the positions of NH₄ and K were reversed by an increase in the concentration of the solutions.

Acids. Seawater to which acid is added, gradually returns to its normal hydrogen-ion concentration. Therefore, the solutions to be tested were freshly made up immediately before being applied to the animal. This was accomplished by having a stock 0.1 N solution made up in rain water, and diluting it to the desired concentrations with seawater. The effect of the dilution of the seawater is insignificant. Three acids, hydrochloric, formic and acetic, were tested. The following table gives the values which were obtained in Exp. VIII.9, typical of the others (table 8).

TABLE 8
Liminal strengths of acids for the stimulation of Ascidia

| ACID | CONCENTRATION |
|----------------------|---------------|
| HCl | 0.0016 N |
| HCOOH | 0.0018 N |
| CH ₃ COOH | 0.010 N |

The order of the stimulating efficiency of the acids is, therefore,

HCl > formic > acetic

Bases. As representatives of this group of substances, I used NH₄OH and NaOH. Exp. VIII.9.1 gave the liminal values shown in table 9. This places them in the order,

NaOH > NH₄OH

TABLE 9

| BASE | CONCENTRATION |
|--------------------|---------------|
| NaOH | 0.010 N |
| NH ₄ OH | 0.015 N |

Sugars. Both glycerin and sucrose did not stimulate until they reached a concentration of 1 M. This quantity of solute, plus the salts of the seawater in which these substances were dissolved, brought the concentration of the stimulating solution to just that equivalent of concentrated seawater which irritated *Ascidia* osmotically. We can, therefore, conclude that *Ascidia* is not sensitive to these two substances.

This has been found to be generally true for aquatic animals (Parker, '12). Crozier ('15a), however, has shown that glycerin and maltose can stimulate *Holothuria*.

Alkaloids. The sulphates of quinine, strychnine and morphine were tested. The order of their effectiveness,

strychnine > quinine > morphine

was found in Exp. VIII.1, the results of which are given in table 10.

These values show a surprising sensitivity of the species to alkaloids. A bitter taste in man may be secured from 0.00004 M quinine sulphate. This amounts to one-tenth of the uncorrected concentration to which *Ascidia* reacts.

TABLE 10

| ALKALOID | CONCENTRATION |
|------------|---------------|
| Strychnine | 0.00005 M |
| Quinine | 0.0004 M |
| Morphine | 0.001 M |

Anesthetics. Ether, chloral hydrated, ethyl alcohol and amyl alcohol all caused reactions which were very pronounced. The order of their effectiveness, taken from the values obtained in Exp. VIII.1.1 and VIII.2.2 and given in the accompanying table (table 11), is

amyl alc. > chloral, ether > ethyl alc.

TABLE 11

| ANESTHETIC | CONCENTRATION |
|------------------|---------------|
| Ether | 0.02 M |
| Chloral hydrated | 0.02 M |
| Ethyl alcohol | 0.75 M |
| Amyl alcohol | 0.001 M |

6. *Nature of the sense organs*

The morphological nature of the chemical, and indeed of any other kind of receptors in ascidians, is practically unknown. Seeliger ('93-'11, p. 323) has described, rather doubtfully and with much reserve, the presence of bristle cells on the tentacles of *Ciona*. Lorleberg ('07); however, failed to secure any trace of such structures in *Styelopsis*; although he found many regions richly supplied with nerve endings. It may then be that the organs of chemical sense in *Ascidia* are similar to those which underlie the common chemical sense of vertebrates (Parker, '12). The physiology of the receptors would seem to favor such an assumption. The problem of the physiological nature of the chemical sense organs is simplified in *Ascidia atra* by the monotony of response to all classes of substances. This negative reaction of the crossed type and its variation with the intensity of the stimulus have already been made clear. We are, therefore, dealing apparently with an automatic reflex, of which the receptor and effector mechanisms are all set, and the conduction

provided for. The application of the stimulating substance to the sense organ merely starts a prearranged process of response.

In order to understand the nature of the process set up in the receptor, it will be necessary to consider more closely the physiological effects of the substances used in the stimulation of *Ascidia*. It was on the basis of the action of the salts of the alkali metals that Höber ('14) first pointed out the relation between irritability and colloidal constitution of the plasma membrane. Since then the ubiquity of the cation and anion series has been demonstrated for such diverse processes as melanophore contraction (Spaeth, '13), hemolysis (Höber, '14) and rhythmic pulsation (Crozier, '16a). The presence of these ionic series in the sensory stimulation of *Ascidia* indicates that the significant process which underlies it, resembles, if it is not identical with, the determining reactions of the other physiological phenomena.

The acids have already received attention in regard to their sensory effects (Richards, '00; Kahlbaum, '00). The anomalies which are exhibited by the acid taste in man are typified in the behavior of the three acids which were used in these experiments. Although HCl is more effective than formic acid, the difference between them is not great. They both, however, are much more powerful than acetic. In the penetration of cells by acids (Crozier, '16b), we find the same order of effectiveness. The anomalies which were referred to are as follows. When the dissociation constants of the acids are taken into account, it is found that the same effect is produced by acetic acid with a lesser quantity of hydrogen ions than by formic acid; and less in turn by formic than by hydrochloric acid. In *Ascidia* the liminal concentrations of the acids contain the following quantities of hydrogen ions: acetic, $4.1 \times 10^{-4}N$; formic, $6.0 \times 10^{-4}N$; and hydrochloric, $1.6 \times 10^{-3}N$.

An analogous difficulty exists in the effects of NaOH and NH_4OH . Experiments on penetration have shown that NH_4OH enters tissue rapidly, whereas NaOH may hardly be said to penetrate living tissue at all. Still, NaOH is more toxic than NH_4OH (Harvey, '13). Similarly it is a more effective sensory stimulant than NH_4OH .

The physiological inertness of the sugars is known only too well to require more than mention. Their ineffectiveness has made their use possible in experiments where the effect of osmotic pressure only is desired (Höber, '14, p. 496). It is therefore altogether in keeping with the parallelism between general physiological activity and sensory stimulation that *Ascidia* fails to be stimulated by even high concentrations of glycerin and sucrose.

It has been suggested that the sensory inactivity of the sugars may be due to the lack of these substances in an aquatic environment (Parker, '12). The improbability of the occurrence of saccharin in the seawater, however, does not prevent its chemical stimulation of *Ascidia*. The liminal concentration of a commercial preparation was 0.025 M, to which the usual negative response was given.

It is necessary, similarly, to look in a different direction for the explanation of the sensitivity of *Ascidia* to alkaloids and anesthetics. The minute quantities of alkaloids which are effective in stimulation find their counterpart in the extremely low concentrations in which they penetrate cells (Overton, '97).

As a consequence of these results there can be no doubt of the essential similarity between the general physiological reactions of chemical substances and their effects on the sensory processes in *Ascidia*. This indicates that the action of the stimulating agent on the sense organ involves an effect of the same nature as the action of these substances on other cells and tissues. Moreover, it shows that the effect of a chemical on the receptor concerns that structure primarily as a cell, and only secondarily as an organ for receiving stimuli.

It must be emphasized that these generalizations are not intended for the sense of taste in vertebrates, but solely for the sensitivity of animals, like *Ascidia* and *Holothuria*, which possess a general chemical sense. This type of irritability corresponds in many ways to the common chemical sense of vertebrates (Parker, '12), although the two need not necessarily be homologous. The problems involved in the higher organs of taste, particularly the sweet taste, do not concern us here. They represent specialization for certain needs; and in the present condition

of our knowledge it is futile to attempt an explanation of their physiology.

It has been tacitly assumed that chemical sense organs are capable of detecting substances in concentrations which fail to affect the ordinary cells of the body. This is largely because the effects on the sense organs become evident through certain effectors, whereas the action on other tissues must be noted by special, indirect means on the cells themselves. When, however other tissues are studied, it is seen that they are influenced by concentrations of the same magnitude as sense cells. The effect of minute changes of the hydrogen and hydroxyl ions on the permeability of eggs and blood corpuscles need only be mentioned. Acids and bases enter cells in concentrations like those which stimulate animals. The poisoning effects of extremely low concentrations of alkaloids are also familiar.

The modifications produced by these various substances are more or less the same for all cells and tissues: witness the similarity of effects produced on egg cells, sperm cells, fronds of algae, blood corpuscles, chromatophores, hearts, medusa bells and a host of others too numerous to mention. The concepts of ionic antagonism and salt balance apply not only to these tissues, but to sensory stimulation as well (Crozier, '15 b). It is therefore clear that chemical sensitivity is merely one of a large number of similar manifestations of the fundamental nature of cells.

The explanation which seems to me to account for all the phenomena of this sensory activity, in *Ascidia* at least, is that the factor which primarily converts a group of cells into chemical sense organs is not any special modification of their structure or sensitivity, but rather their connection, directly or indirectly, with an effector system.

In this way the problem of the chemical sense of such aquatic forms is linked with the general problems of the physical chemistry of cells and tissues. Our present knowledge, in this respect, of the chemical senses is, however, extremely meager. The time is therefore not ripe for any adequate explanation of the process in the receptor cell which results from the contact with a substance in solution.

One such attempt has been made. On the basis of his work on echinoderm eggs and *Arenicola* larvae, Lillie ('11) has proposed an explanation for general irritability. It is, that sensory stimulation means an increase in the permeability of the irritable element.

Lillie's explanation is based on the assumption that the demarcation current and kindred phenomena are functions of the differential permeability of the cell membrane to certain substances, notably H and OH ions. The work of Loeb and Beutner ('14) has, however, shown that this bioelectric potential is due on the contrary to the presence of certain lipoid materials in the protoplasm. It is still uncertain to what extent differential solubility and the effect of interphase boundaries are concerned in the interpretation of these results. It is much to be regretted that the experiments were discontinued.

There is, moreover, another and more significant objection to Lillie's idea. All the substances which increase permeability undoubtedly do stimulate. But many substances, like Ca and the anesthetics in general, all of which have a decreasing action on permeability (Osterhout, '16), also serve as vigorous stimulants to *Ascidia* and other aquatic organisms.

The theory in its present form can therefore not be accepted as an adequate explanation. However, the attempt at an interpretation along the lines of permeability and similar concepts is entirely in the right direction.

VI. SUMMARY

1. *Ascidia* possesses six distinct reactions to stimuli, all of them negative in character. They may be divided into two groups of three each: the direct reflexes, which depend upon a stimulation of the exterior of the body, and the crossed reflexes, which depend upon a stimulation of the interior of the body.

2. The intersiphonal ganglion connects the two siphons. Severing this nervous mass completely abolishes the crossed reactions, and interferes with the direct ones. Nevertheless, each portion of the animal is able to perform its part of a reaction, even though nervously isolated from the rest.

3. *Ascidia* is sensitive to tactile stimulation. The regions of greatest sensitivity are the siphon rims and the oral tentacles.

4. Vibrations through solid and liquid media affect *Ascidia*, although transmission through the seawater is the normal method of stimulation. The receptors are located in the lobes of the siphon rims.

5. The records of the amplitude of contraction to regularly repeated mechanical stimulation show that the cessation of response after a time is due mainly to a fatigue of the receptor mechanism.

6. The 'ocelli' of *Ascidia* are not organs for photo-reception. The animals are sensitive to light of very high intensity only, and the sense organs are located within the siphon near the oral tentacles.

7. *Ascidia* is thermosensitive. It reacts to temperatures above 32°C. and below 20°C.

8. Its test is insensitive to light, heat and chemicals.

9. The animals react to large changes in osmotic pressure, and to the presence of the following classes of substances in solution: salts, acids, bases, alkaloids and anesthetics. Solutions of sugars do not stimulate, but saccharin gives a decided reaction.

10. The liminal concentrations and the relative effectiveness of all these stimulating substances are very similar to those which have been demonstrated for other physiological activities. It is, therefore, suggested that the primary factor which converts a group of cells of *Ascidia* into chemical sense organs is their connection with an effector system.

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CHANGES IN THE RELATIVE WEIGHTS OF THE VARIOUS PARTS, SYSTEMS AND ORGANS OF YOUNG ALBINO RATS UNDERFERED FOR VARIOUS PERIODS

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ONE FIGURE AND FOUR TABLES

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It has recently been shown that although growth in body weight in the young rat may be completely suppressed by underfeeding

for considerable periods, nevertheless, certain organs and parts of the body having a very strong growth tendency continue to increase in weight, other organs and parts with a weaker growth capacity remain nearly unchanged, while still other parts suffer a loss in weight (Jackson '15 b and Stewart '16). These changes were observed chiefly in rats whose underfeeding began at the age of three weeks and terminated at the age of ten or twelve weeks. Very few observations have been recorded, however, concerning such changes which may occur either in younger animals subjected to inanition for various periods, or in animals underfed for much longer periods. An investigation was therefore undertaken in order to determine these changes. The work was done in the Institute of Anatomy of the University of Minnesota, under the supervision of Dr. C. M. Jackson, to whom I am indebted for valuable aid and direction.

MATERIAL AND METHODS

For the present investigation eighty-nine albino rats (*Mus norvegicus albinus*) were used (table 1), all of which were autopsied at the close of the experiment. They included thirty-four controls and fifty-five test rats.

From the litters used, the controls were selected at the beginning of the experiment, the sex being determined according to the method of Jackson ('12). Seven (4 M, 3 F) of the controls were killed and autopsied when they had reached (on full feeding) an approximate average net body weight of 10 grams, four (1 M, 1 F) at 13 grams, fourteen (7 M, 7 F) at 15 grams, one (M) at 27 grams, one (F) at 40 grams, four (2 M, 2 F) at 50 grams, and three (2 M, 1 F) at 70 grams. Whenever possible, the controls were selected from the same litters as the test rats. In some instances, however, this was not possible, especially in the case of the controls for the rats underfed for very long periods.

The majority of the test rats were starved for intermittent periods starting a short time (24 to 48 hours) after birth, and were killed (by chloroform) and autopsied at the age of three weeks (17 rats; 9 M, 8 F), six weeks (9 rats; 6 M, 3 F), and ten weeks

TABLE 1

Average and range of gross and net body weight, body length, tail ratio, and weights of the head, trunk, extremities, skeleton, musculature visceral group and remainder
 Test rats in first six groups were underfed from birth; last six groups from age of three weeks

| DESCRIPTION OF RATS, NUMBER, SEX AND AGE | GROSS BODY WEIGHT <i>grams</i> | NET BODY WEIGHT <i>grams</i> | BODY LENGTH <i>mm.</i> | RATIO OF TAIL LENGTH TO BODY LENGTH |
|---|-----------------------------------|---------------------------------|---------------------------|--|
| 4 Control M 7 5(7-8) da. | 10.6(10.0-11.2) | 9.82(9.3-10.3) | 65.5(64.0-67.0) | 46.9(40.6-56.7) |
| 9 Test M 21.2(20-23) da. | 10.2(9.5-11.5) | 9.90(9.1-11.2) | 68.0(66.0-69.0) | 59.0(57.4-63.6) |
| 3 Control F 7 da. | 10.5(10.0-10.8) | 9.77(9.3-10.1) | 64.7(62.0-66.0) | 45.4(43.9-47.0) |
| 8 Test F 22.2(21-28) da. | 10.5(9.5-11.5) | 10.20(9.1-10.9) | 69.8(66.0-76.0) | 66.3(52.9-71.2) |
| 3 Control M 14(13-17) da. | 13.6(13.5-13.6) | 12.97(12.6-13.3) | 71.0(70.0-72.0) | 57.2(56.3-58.3) |
| 6 Test M 47(42-59) da. | 14.1(13.1-15.9) | 12.50(11.3-13.3) | 82.3(77.0-87.0) | 68.2(67.1-69.0) |
| 1 Control F 13 da. | 13.2 | 12.76 | 71.0 | 60.6 |
| 3 Test F 42 da. | 13.8(13.0-14.9) | 12.82(12.2-13.7) | 78.3(76.0-80.0) | 75.3(74.7-76.3) |
| 7 Control M 12.6(11-15) da. | 15.9(15.0-16.9) | 14.95(14.2-15.9) | 75.2(71.0-79.0) | 53.7(47.9-56.6) |
| 6 Test M 69.2(64-72) da. | 15.5(14.4-17.0) | 14.29(13.4-15.6) | 86.8(83.0-89.0) | 73.9(62.4-85.5) |
| 7 Control F 12.9(11-15) da. | 15.6(15.0-16.2) | 14.71(13.8-15.7) | 75.3(74.0-77.0) | 56.6(53.9-60.8) |
| 7 Test F 69.1(67-72) da. | 16.1(14.0-17.1) | 14.86(13.2-15.7) | 84.9(81.0-88.0) | 82.0(76.1-87.7) |
| 1 Control M 22 da. | 29.6 | 27.25 | 98.0 | 73.5 |
| 1 Test M 139 da. | 30.0 | 28.67 | 118.0 | 89.0 |
| 1 Control F 37 da. | 44.5 | 39.99 | 116.0 | 78.4 |
| 1 Test F 316 da. | 42.4 | 39.63 | 130.0 | |
| 2 Control M 36(35-37) da. | 51.6(50.5-52.8) | 49.10(47.5-50.7) | 120.5(120.0-121.0) | 65.5(65.2-65.8) |
| 4 Test M 412(374-428) da. | 48.6(44.1-52.4) | 46.02(40.9-49.9) | 134.3(132.0-140.0) | 88.3(84.1-93.2) |
| 2 Control F 44(43-45) da. | 53.3(50.0-56.5) | 48.55(45.2-51.9) | 122.5(121.0-124.0) | 83.7(83.5-83.9) |
| 4 Test F 392(377-428) da. | 54.1(49.0-56.5) | 50.41(45.3-53.9) | 138.5(135.0-148.0) | 93.1(89.2-97.1) |
| 2 Control M 47(46-48) da. | 76.5(76.0-77.0) | 71.93(70.5-73.4) | 140.5(140.0-141.0) | 78.95(72.1-85.8) |
| 4 Test M 291(223-316) da. | 77.6(66.8-101.5) | 69.87(63.4-83.2) | 152.8(150.0-157.0) | 92.70(87.9-94.8) |
| 1 Control F 53 da. | 73.0 | 67.85 | 137.0 | 79.60 |
| 2 Test F 314(311-316) da. | 81.6(67.0-96.2) | 67.20(58.1-76.3) | 149.0(146.0-152.0) | 92.25(91.1-93.4) |

TABLE 1—Continued

| HEAD | UPPER EXTREMITIES | LOWER EXTREMITIES | TRUNK | INTEGUMENT |
|------------------|---------------------------------|------------------------------------|------------------------------------|------------------------------------|
| <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> |
| 2.35(2.20-2.50) | 0.686(0.454-0.814) | 0.930(0.684-1.090) | 5.856(5.305-6.558) | 1.999(1.800-2.109) |
| 2.78(2.60-2.90) | 0.797(0.734-0.912) | 1.191(1.089-1.294) | 5.130(4.492-6.321) | 2.095(1.553-2.760) |
| 2.51(2.44-2.60) | 0.668(0.494-0.806) | 0.940(0.650-1.101) | 5.648(4.892-6.548) | 2.124(1.940-2.279) |
| 2.88(2.70-3.00) | 0.804(0.697-0.901) | 1.237(1.036-1.371) | 5.285(4.630-5.962) | 2.032(1.407-2.000) |
| 3.40(3.30-3.50) | 1.168(1.120-1.204) | 1.596(1.565-1.657) | 6.839(6.564-7.114) | 3.181(2.920-3.450) |
| 3.30(3.10-3.50) | 0.928(0.855-0.992) | 1.649(1.588-1.767) | 6.635(5.625-7.437) | 1.921(1.714-2.116) |
| 3.40 | 1.176 | 1.606 | 6.578 | 3.340 |
| 3.23(2.90-3.50) | 0.918(0.891-0.959) | 1.626(1.465-1.751) | 7.041(6.657-7.513) | 1.784(1.578-1.925) |
| 3.70(3.50-3.90) | 1.208(1.000-1.306) | 1.842(1.611-2.000) | 8.209(7.644-8.945) | 3.895(3.540-4.700) |
| 3.80(3.50-4.10) | 1.045(0.917-1.137) | 1.899(1.690-2.012) | 7.564(6.834-8.505) | 2.064(1.650-2.318) |
| 3.70(3.40-3.80) | 1.194(1.000-1.325) | 1.869(1.710-2.000) | 7.965(7.150-8.722) | 3.909(3.414-4.500) |
| 3.30(3.80-4.10) | 1.079(0.912-1.262) | 1.970(1.771-2.143) | 7.913(6.417-8.712) | 2.018(1.872-2.534) |
| 5.10 | 2.210 | 4.167 | 15.771 | 4.730 |
| 6.00 | 2.100 | 4.600 | 15.966 | 4.400 |
| 6.40 | 3.105 | 6.900 | 23.585 | 7.118 |
| 8.00 | 2.992 | 6.095 | 22.547 | 6.515 |
| 6.90(6.7-7.1) | 3.611(3.600-3.622) | 7.534(7.369-7.700) | 31.054(29.809-32.300) | 9.413(9.400-9.426) |
| 8.70(7.4-9.3) | 3.477(2.587-3.911) | 6.998(5.576-7.889) | 26.842(25.357-28.843) | 7.449(5.361-8.900) |
| 6.85(6.4-7.3) | 3.487(3.280-3.693) | 7.697(7.240-8.154) | 30.496(28.253-32.739) | 9.231(8.820-9.641) |
| 8.75(8.4-9.2) | 3.816(3.400-4.199) | 8.180(6.823-9.502) | 29.662(26.386-34.161) | 9.174(8.164-10.000) |
| 8.75(8.7-8.8) | 4.964(4.798-5.130) | 11.577(11.524-11.630) | 46.665(45.478-47.852) | 14.055(12.334-15.777) |
| 10.55(9.8-11.2) | 6.033(5.200-6.900) ¹ | 14.200(12.800-15.200) ¹ | 39.400(32.100-50.500) ¹ | 17.300(11.208-22.200) ¹ |
| 8.60 | 5.096 | 11.757 | 42.392 | 12.713 |
| 10.35(10.1-10.6) | 5.550(4.800-6.300) | 13.700(11.600-15.800) | 37.600(31.600-43.600) | 18.700) ⁴ |

TABLE 1—Continued

| LIGAMENTOUS SKELETON | MOIST CARTILAGINOUS SKELETON | DRY CARTILAGINOUS SKELETON | MUSCULATURE | VISCERAL GROUP | REMAINDER |
|------------------------------------|---------------------------------|---------------------------------|------------------------------------|------------------------------------|---------------------------------|
| grams | grams | grams | grams | grams | grams |
| 1.636(1.393-1.799) | 0.715(0.481-0.959) ¹ | 0.145(0.118-0.184) ¹ | 2.244(1.590-2.473) | 1.977(1.891-2.153) | 1.964(1.664-2.686) |
| 1.915(1.767-2.092) | 1.610(1.398-1.887) | 0.359(0.290-0.451) ² | 2.442(2.197-2.986) | 2.501(2.271-2.719) | 1.076(0.534-2.027) ⁸ |
| 1.598(1.509-1.700) | 0.856(0.543-1.168) ³ | 0.136 ⁴ | 2.356(2.020-2.745) | 2.002(1.896-2.135) | 1.683(1.371-2.137) |
| 1.923(1.610-2.256) | 1.440(1.216-1.897) | 0.389(0.328-0.492) ² | 2.530(1.640-2.890) | 2.606(2.322-2.982) | 1.109(0.583-1.554) |
| 2.302(2.225-2.394) | 1.699(1.519-1.865) | 0.329(0.289-0.359) | 3.053(2.953-3.119) | 2.720(2.568-2.956) | 1.705(1.184-2.129) |
| 2.686(2.536-2.867) | 2.331(1.891-2.485) | 0.554(0.483-0.753) | 3.338(2.979-3.608) | 3.512(3.184-3.905) | 1.104(0.000-2.038) |
| 2.297 | 1.815 | 0.342 | 3.190 | 2.787 | 1.146 |
| 2.599(2.386-2.715) | 2.350(2.308-2.418) | 0.526(0.422-0.630) | 3.659(3.510-3.860) | 3.563(3.400-3.745) | 1.213(0.622-1.695) |
| 2.372(2.130-2.714) | 1.667(1.399-2.088) ⁶ | 0.376(0.336-0.423) ¹ | 3.840(3.609-4.060) ⁵ | 2.935(2.775-3.042) | 1.877(1.686-2.131) ⁵ |
| 2.937(2.796-3.153) | 2.568(2.121-2.737) | 0.767(0.662-0.856) ⁵ | 3.946(3.368-4.467) | 3.840(3.425-4.209) | 1.486(1.028-2.088) |
| 2.341(2.204-2.561) ⁵ | 1.528(1.294-2.006) ⁷ | 0.372(0.350-0.390) ⁷ | 3.863(3.488-5.126) ⁵ | 2.935(2.758-3.168) | 1.694(0.105-2.564) ⁵ |
| 2.894(2.700-3.013) | 2.386(1.948-2.847) | 0.731(0.683-0.856) | 4.475(3.660-5.227) | 4.204(3.768-5.065) | 1.271(0.855-1.703) |
| 4.305 | 3.587 | 2.079 | 8.175 | 5.453 | 4.585 |
| 5.400 | 4.300 | | 8.500 | 5.388 | 4.978 |
| 5.844 | | | 14.448 | 7.448 | 5.132 |
| 8.838 | 7.957 | 3.081 | 12.393 | 7.668 | 4.220 |
| 6.785(6.620-6.951) | 5.572(5.411-5.733) | 1.603(1.336-1.870) | 16.650(16.320-16.980) | 8.605(8.298-8.912) | 7.651(5.900-9.402) |
| 8.151(7.558-8.796) | 7.209(6.403-8.341) | 3.523(2.897-4.035) | 16.377(11.472-18.821) | 8.893(7.934-11.283) | 5.146(4.074-5.734) |
| 6.207(6.066-6.347) | 5.193 ⁴ | 3.912(3.340-4.415) | 16.447(15.684-17.209) | 9.646(8.935-10.356) | 7.000(5.668-8.333) |
| 8.963(7.930-10.111) | 7.774(7.055-8.813) ¹ | | 18.110(13.355-21.076) | 9.537(7.026-13.744) | 4.626(3.466-5.894) |
| 8.457(7.903-9.011) | 7.197(6.281-8.104) | 2.503(2.229-2.776) | 28.082(26.474-29.690) | 13.680(13.025-14.336) | 7.662(5.129-10.196) |
| 12.500(11.300-13.800) ¹ | 9.030(8.800-9.300) ¹ | 4.273(3.738-4.890) | 33.700(30.000-37.400) ³ | 11.186(10.733-11.639) ⁵ | 6.300 ⁴ |
| 8.202 | 7.232 | | 26.221 | 10.254 | 10.455 |
| 11.550(11.100-12.000) | 9.400(9.100-9.700) | 3.856(3.692-4.020) | 35.300 ⁴ | 10.698 ⁴ | 0.000 |

¹ Average of 3 individuals.² Average of 7 individuals.³ Average of 2 individuals.⁴ Average of 1 individual.⁵ Average of 6 individuals.⁶ Average of 5 individuals.⁷ Average of 4 individuals.⁸ Average of 8 individuals.

(13 rats; 6 M, 7 F), the average net body weight at each age being approximately 10, 13 and 15 grams respectively.

In addition sixteen rats, starting at three weeks of age, were underfed for much longer periods (to 139 to 412 days of age; see table 1). They were then killed and autopsied. For one litter of the group fasting for a long period (M 29), the underfeeding started at the age of five weeks. Four rats of litters S 26 and M 29 (one male and one female of each litter), and one male of litter St 44 were accidentally asphyxiated by illuminating gas. These rats are all included in the two groups of test rats weighing approximately 70 grams. The two asphyxiated individuals of litter M 29 had been refed about thirty-six hours, which has resulted chiefly in a great increase in the contents of the alimentary canal.

In general the plan was to kill the test rats at the same body weight as the corresponding controls, but this was not always possible. Nevertheless, the average body weight of the different groups of test rats differs only slightly from that for the corresponding controls, as is shown in table 1. In comparing the data from the test rats with those from the controls throughout the paper, this difference has generally been disregarded, although strictly speaking, there should be a slight correction in every case for differences in body weight. Such a correction should be based upon the net body weight (excluding content of stomach and intestines), rather than upon the gross body weight, however. As the differences in body weight are in all cases small, it seems justified to ignore them in making comparisons.

The control rats remained constantly with the mother throughout the nursing period (three weeks). After this time they were fed an abundant diet of whole wheat (Graham) bread soaked in whole milk. Water was also supplied.

The test rats (in the experiments upon very young animals) were removed from the mother at frequent intervals for periods of usually 15 to 24 hours (occasionally longer, the maximum being 43 hours), and were permitted to nurse during the intervening time. For convenience, these test rats are frequently referred to throughout this paper as the rats fasting from birth

for various periods. The total number of hours that the test rats of a litter were separated from the mother during the first three weeks (504 hours) after birth averaged 260 hours (213 to 285 hours) or more than half of the total period. Brüning ('14) was able to isolate young rats from the mother for only about one-fourth of the total nursing period.

In most instances when young fasting rats were returned to the nest, the mother would immediately take care of them. Occasionally, however, after being disturbed, the mother would abandon her litter entirely. In these cases it was often possible to save the young rats by putting them to nurse with other mothers whose young were fasting at that time. By continually putting young rats of different litters in the nest, it was found possible to keep one female nursing for twelve weeks.

The age of weaning for the albino rat is usually given as three weeks. However, Donaldson ('15) ('The Rat,' p. 19) states that the young, if permitted, will continue to depend partly on the mother for some days longer. One of my litters was observed to continue nursing until six weeks of age.

When three weeks old, the test rats underfed from birth, and also those used in the experiments starting at three weeks, were placed on the bread and milk diet, receiving only a limited and carefully measured quantity daily. As was observed by Jackson ('15 b) and Stewart ('16), the young rats in the present experiments were kept at maintenance for a considerable period upon a gradually diminishing ration. Thus for one litter (St 96) during 30 days underfeeding the daily amount of food required for maintenance of body weight at approximately 14.5 grams decreased from an average of 3.87 grams to 2.97 grams per rat. Later, however, the maintenance ration apparently becomes more nearly constant. For example, in the case of another litter (St 46) underfed for a very long period the total daily weight of food consumed by 5 rats from the 131st to the 180th day of the experiment was constantly 33 grams. The average body weight of these rats during this period remained unchanged (47.6 grams.) Stewart ('16) for one litter found no decrease in the maintenance ration from the 60th to the 120th day of the experiment.

The decrease in the maintenance ration noted in the young stunted rats is perhaps associated with the decrease in the intensity of metabolism which normally occurs with advancing age. Jackson ('15 b) suggests that during maintenance in young animals the amount of living protoplasm may be actually reduced, being replaced by water absorption, or that the food-intake may be more economically utilized under these conditions. He also mentions the decrease in body temperature as a possible factor. This problem is apparently of sufficient interest and importance to warrant a more exact and thorough investigation from the physiological point of view.

Since it was found very difficult to hold the test rats strictly at constant body weight and keep them alive for very long periods, a slight increase in body weight was usually permitted. Aron ('11), Jackson ('15 b) and Stewart ('16) similarly found it increasingly difficult to hold animals at constant body weight as the experiment progressed.

Separate weight records were kept for each rat, the individuals being identified by staining the integument with an alcoholic solution of picric acid. The identification marks had to be renewed morning and evening on the very young rats, but after the appearance of hairs the stains were very permanent. The young test rats were weighed daily immediately before feeding, whereas the controls, and also the test rats underfed for very long periods, were weighed at gradually increasing intervals as they grew older (about once in two weeks when about three months of age).

The cages used, and also the warm room in which the test rats were kept while fasting, have been described by Stewart ('16).

The autopsy technique employed by Jackson and Lowrey ('12) and Jackson ('13) was used with but few modifications. The various organs and parts were placed in a moist chamber when removed from the animal, and were weighed in a closed container on balances accurate to one-tenth milligram (0.0001 gram).

The data collected for the controls in this work were carefully compared with the published records of Jackson and Lowrey ('12), Jackson ('13 and '15), Hatai ('13 and '14), King ('15), and

with the Wistar norm tables of Donaldson ('15). By this means it was possible to detect any marked variations from the normal in my control rats. For purposes of convenient comparison, the weights of organs from the Wistar tables for normal rats of body length corresponding to my controls have been inserted in table 2. In general, it will be noted that both the body weight and the corresponding organ weights are slightly higher in my controls than in the Wistar tables. If the comparison were based upon body weight rather than body length, there would usually be much closer agreement. While body length is a satisfactory basis for comparison between my controls and the Wistar data, it cannot be used in the case of the test rats, for as will appear later, the body length in underfed rats continues to increase even when the body weight is held constant. Body weight therefore was selected as a basis for comparison between my controls and test rats, since it can easily be held constant, which is not true for the body length.

In the present paper, for the most part only average data are published. However, a copy of the original individual observations will be deposited in The Wistar Institute of Anatomy and Biology, Philadelphia, where they may be consulted by those interested.

The normal variability of the various organs makes it necessary in some cases to exercise considerable care in drawing conclusions from the relatively limited number of observations. Nevertheless, the data appear sufficient to establish fairly accurately some of the more obvious and important changes due to the experiment.

An abstract of that part of the present investigation dealing with the underfeeding of the rats from birth to three, six and ten weeks of age was published in the Proceedings of the American Association of Anatomists, New York meeting, December, 1916 (Stewart, '17).

TABLE 2

Average (and range) of weight of the organs, with data from the Wistar tables (Donaldson, '15) for rats of body length corresponding to my controls. (In case of the thymus age is the basis of comparison)

Test rats in the first six groups were underfed from birth; last six groups from age of three weeks

| DESCRIPTION OF RATS, NUMBER, SEX AND AGE | BODY LENGTH | GROSS BODY WEIGHT | NET BODY WEIGHT | BRAIN | SPINAL CORD |
|---|-------------------------|-------------------------|--------------------|-----------------------------|-----------------------------|
| | mm. | grams | grams | grams | grams |
| 4 Control M 7.5(7-8)da. Wistar data | 65.5(64.0-67.0) 65.0 | 10.6(10.0-11.2) 9.4 | 9.82(9.28-10.33) | 0.609(0.577-0.634) 0.650 | 0.081(0.076-0.087) 0.071 |
| 9 Test M 21(20-23)da. | 68.0(66.0-69.0) | 10.2(9.5-11.5) | 9.90(9.12-11.20) | 0.975(0.839-1.034) | 0.137(0.114-0.157) |
| 3 Control F 7 da. Wistar data | 64.7(62.0-66.0) 65.0 | 10.5(10.0-10.8) 9.9 | 9.77(9.26-10.13) | 0.625(0.608-0.644) 0.679 | 0.079(0.077-0.082) 0.077 |
| 8 Test F 22(21-28)da. | 69.8(66.0-76.0) | 10.5(9.5-11.5) | 10.20(9.13-10.88) | 0.996(0.934-1.035) | 0.135(0.105-0.164) |
| 3 Control M 14(13-17)da. Wistar data | 71.0(70.0-72.0) 71.0 | 13.6(13.5-13.6) 11.8 | 12.97(12.61-13.28) | 0.962(0.907-1.025) 0.840 | 0.124(0.109-0.136) 0.091 |
| 6 Test M 47(42-59)da. | 82.3(77.0-87.0) | 14.2(13.1-15.9) | 12.50(11.34-13.27) | 1.045(0.972-1.088) | 0.176(0.153-0.186) |
| 1 Control F 13 da. Wistar data | 71.0 71.0 | 13.2 12.5 | 12.76 | 1.035 0.876 | 0.145 0.098 |
| 3 Test F 42 da. | 78.3(76.0-80.0) | 13.8(13.0-14.9) | 12.82(12.21-13.72) | 1.005(0.923-1.059) | 0.184(0.160-0.196) |
| 7 Control M 13(11-15)da. Wistar data | 75.2(73.0-79.0) 75.0 | 15.9(15.0-16.9) 13.6 | 14.95(14.24-15.95) | 1.021(0.963-1.087) 0.952 | 0.119(0.100-0.129) 0.104 |
| 6 Test M 69(64-72)da. | 86.8(83.0-89.0) | 15.5(14.4-17.0) | 14.29(13.45-15.57) | 1.073(0.961-1.145) | 0.199(0.174-0.211) |

TABLE 2.—Continued

| | | | | | |
|--|-----------------------------|-------------------------|-----------------------------|-----------------------------|-----------------------------|
| 7 Control F 13(11-15)da. Wistar data | 75.3(74.0-76.0) 75.0 | 15.6(15.0-16.2) 14.3 | 14.71(13.84-15.73) 0.974 | 1.016(0.950-1.086) 0.112 | 0.121(0.103-0.133) 0.112 |
| 7 Test F 69(67-72)da. | 84.9(81.0-88.0) | 16.1(14.0-17.1) | 14.86(13.20-15.67) | 1.117(1.084-1.180) | 0.207(0.188-0.225) |
| 1 Control M 22 da. Wistar data | 98.0 98.0 | 29.6 26.2 | 27.25 | 1.357 1.271 | 0.198 0.182 |
| 1 Test M 139 da. | 118.0 | 30.0 | 28.67 | 1.418 | 0.285 |
| 1 Control F 37 da. Wistar data | 116.0 116.0 | 44.5 42.2 | 39.99 | 1.444 1.411 | 0.262 0.261 |
| 1 Test F 316 da. | 130.0 | 42.4 | 39.63 | 1.636 | 0.409 |
| 2 Control M 36(35-37)da. Wistar data | 120.5(120.0-121.0) 121.0 | 51.6(50.5-52.8) 44.4 | 49.10(47.5-50.7) | 1.554(1.521-1.588) 1.448 | 0.300(0.296-0.303) 0.265 |
| 4 Test M 412(374-428)da. | 134.3(132.0-140.0) | 48.6(44.1-52.4) | 46.02(40.9-49.9) | 1.570(1.496-1.664) | 0.407(0.394-0.424) |
| 2 Control F 44 (43-45)da. Wistar data | 122.5(121.0-124.0) 123.0 | 53.3(50.0-56.5) 49.1 | 48.55(45.2-51.9) | 1.427(1.350-1.505) 1.45 | 0.282(0.276-0.287) 0.287 |
| 4 Test F 392(377-428)da. | 138.5(135.0-148.0) | 54.1(49.0-56.5) | 50.41(45.3-53.9) | 1.533(1.516-1.549) | 0.408(0.372-0.468) |
| 2 Control M 47(46-48)da. Wistar data | 140.5(140.0-141.0) 141.0 | 76.5(76.0-77.0) 66.7 | 71.95(70.5-73.4) | 1.500(1.429-1.571) 1.569 | 0.322(0.294-0.351) 0.338 |
| 4 Test M 201(223-316)da. | 152.8(150.0-157.0) | 77.6(66.8-101.5) | 69.79(63.4-83.2) | 1.576(1.502-1.626) | 0.415(0.363-0.466) |
| 1 Control F 53 da. Wistar data | 137.0 137.0 | 73.0 65.5 | 67.85 | 1.487 1.540 | 0.312 0.341 |
| 2 Test F 314(311-316)da | 149.0(146.0-152.0) | 81.6(67.0-96.2) | 67.20(58.1-76.3) | 1.540(1.454-1.626) | 0.363 ^s |

TABLE 2—Continued

| EXTRALLIS | THYROID | THYMUS | HEART | LUNGS | LIVER |
|---------------------------------|------------------------------------|-----------------------|--------------------|--------------------|-----------------|
| <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> |
| 0.065(0.058-0.070) | 0.0030(0.0026-0.0034) | 0.023(0.018-0.027) | 0.074(0.064-0.086) | 0.201(0.172-0.225) | 0.37(0.33-0.42) |
| 0.058 | 0.0026 | 0.020 | 0.066 | 0.127 | 0.45 |
| 0.090(0.081-0.097) | 0.0030(0.0020-0.0036) | 0.015(0.0069-0.027) | 0.066(0.058-0.080) | 0.147(0.129-0.163) | 0.40(0.36-0.44) |
| 0.064(0.062-0.067) | 0.0027(0.0026-0.0029) | 0.023(0.020-0.025) | 0.073(0.063-0.084) | 0.191(0.178-0.205) | 0.38(0.35-0.41) |
| 0.061 | 0.0028 | 0.020 | 0.069 | 0.131 | 0.48 |
| 0.094(0.083-0.098) | 0.0030(0.0017-0.0053) | 0.017(0.011-0.029) | 0.074(0.063-0.090) | 0.141(0.126-0.171) | 0.48(0.37-0.63) |
| 0.087(0.084-0.091) | 0.0042(0.0032-0.0054) | 0.037(0.031-0.040) | 0.081(0.073-0.090) | 0.210(0.197-0.235) | 0.45(0.37-0.53) |
| 0.069 | 0.0033 | 0.031 | 0.083 | 0.148 | 0.71 |
| 0.118(0.106-0.127) | 0.0041(0.0018-0.0050) ¹ | 0.012(0.0050-0.020) | 0.092(0.082-0.112) | 0.149(0.126-0.169) | 0.71(0.53-0.96) |
| 0.092 | 0.0046 | 0.037 | 0.084 | 0.195 | 0.40 |
| 0.072 | 0.0034 | 0.029 | 0.087 | 0.154 | 0.79 |
| 0.118(0.110-0.124) | 0.0047(0.0035-0.0060) | 0.018(0.012-0.025) | 0.095(0.090-0.104) | 0.144(0.132-0.162) | 0.83(0.78-0.89) |
| 0.093(0.088-0.097) | 0.0040(0.0028-0.0056) | 0.048(0.049-0.056) | 0.094(0.085-0.110) | 0.230(0.207-0.272) | 0.52(0.47-0.58) |
| 0.076 | 0.0037 | 0.029 | 0.095 | 0.163 | 0.91 |
| 0.149(0.131-0.157) | 0.0038(0.0031-0.0049) | 0.0082(0.0051-0.0150) | 0.109(0.094-0.131) | 0.183(0.141-0.293) | 0.82(0.69-1.00) |
| 0.093(0.087-0.098) | 0.0040(0.0032-0.0052) | 0.048(0.041-0.057) | 0.089(0.083-0.098) | 0.238(0.216-0.263) | 0.56(0.50-0.62) |
| 0.078 | 0.0039 | 0.029 | 0.099 | 0.169 | 0.98 |
| 0.160(0.158-0.161) ² | 0.0045(0.0031-0.0064) | 0.011(0.0050-0.018) | 0.124(0.106-0.149) | 0.165(0.132-0.258) | 0.95(0.74-1.10) |

TABLE 2—Continued

| | | | | | |
|---------------------------------|------------------------------------|----------------------|---------------------|---------------------------------|-----------------|
| 0.124 | 0.0064 | 0.063 | 0.197 | 0.275 | 1.26 |
| 0.109 | 0.0066 | 0.051 | 0.168 | 0.254 | 2.05 |
| 0.230 | 0.0041 | 0.008 | 0.150 | 0.269 | 1.08 |
| 0.159 | 0.0072 | 0.125 | 0.211 | 0.345 | 1.92 |
| 0.136 | 0.0097 | 0.109 | 0.246 | 0.353 | 3.13 |
| 0.233 | 0.0050 | lost | 0.299 | 1.536* | 1.40 |
| 0.161(0.161-0.162) | 0.0137(0.0118-0.0157) | 0.207(0.181-0.232) | 0.335(0.333-0.338) | 0.454(0.424-0.485) | 2.06(1.97-2.15) |
| 0.140 | 0.0101 | 0.104 | 0.256 | 0.366 | 3.26 |
| 0.287(0.262-0.314) | 0.0067(0.0054-0.0081) | 0.011(0.0078-0.0164) | 0.0263(0.242-0.276) | 0.659(0.567-0.835) ³ | 2.04(1.66-2.60) |
| 0.164(0.158-0.171) | 0.0074(0.0071-0.0077) | 0.119(0.115-0.122) | 0.276(0.258-0.293) | 0.371(0.364-0.379) | 2.79(2.65-2.94) |
| 0.146 | 0.0110 | 0.144 | 0.276 | 0.393 | 3.53 |
| 0.276(0.235-0.297) | 0.0074(0.0044-0.0108) | 0.022(0.010-0.037) | 0.286(0.238-0.371) | 0.401(0.395-0.407) ⁴ | 2.46(1.54-4.15) |
| 0.173(0.165-0.181) | 0.0142(0.0106-0.0178) | 0.275(0.250-0.299) | 0.380(0.369-0.391) | 0.474(0.456-0.493) | 4.19(3.57-4.81) |
| 0.166 | 0.0139 | 0.160 | 0.349 | 0.492 | 4.47 |
| 0.277(0.277-0.278) ⁴ | 0.0085(0.0076-0.0090) ³ | 0.0258(0.021-0.039) | 0.350(0.312-0.420) | 0.559(0.447-0.705) | 2.56(2.03-3.46) |
| 0.184 | 0.0070 | 0.228 | 0.344 | 0.473 | 3.15 |
| 0.165 | 0.0137 | 0.192 | 0.344 | 0.485 | 4.41 |
| 0.125 ⁵ | 0.0075 ⁵ | 0.037(0.035-0.038) | 0.366(0.340-0.393) | 0.616(0.548-0.685) ⁶ | 2.75(1.84-3.66) |

TABLE 2.—Continued

| SPLEEN | STOMACH AND INTESTINES | | SUPRARENALS | KIDNEYS |
|--------------------|------------------------|-----------------------|------------------------------------|--------------------|
| | Full <i>grams</i> | Empty <i>grams</i> | | |
| 0.053(0.032-0.074) | 1.123(1.015-1.284) | 0.34(0.29-0.41) | 0.0027(0.0022-0.0032) | 0.125(0.111-0.138) |
| 0.027 | | 0.29 | 0.0041 | 0.143 |
| 0.026(0.020-0.032) | 0.773(0.668-0.935) | 0.43(0.35-0.53) | 0.0038(0.0032-0.0045) | 0.142(0.120-0.164) |
| 0.048(0.042-0.059) | 1.080(1.032-1.148) | 0.38(0.32-0.46) | 0.0027(0.0024-0.0030) | 0.130(0.121-0.138) |
| 0.029 | | 0.31 | 0.0044 | 0.151 |
| 0.026(0.018-0.039) | 0.875(0.597-1.163) | 0.46(0.34-0.64) | 0.0050(0.0041-0.0070) | 0.167(0.136-0.223) |
| 0.043(0.040-0.048) | 1.114(0.837-1.463) | 0.52(0.47-0.56) | 0.0042(0.0032-0.0050) | 0.153(0.127-0.168) |
| 0.036 | | 0.55 | 0.0052 | 0.179 |
| 0.057(0.034-0.108) | 2.481(1.746-3.923) | 0.83(0.70-1.05) | 0.0074(0.0066-0.0092) | 0.223(0.186-0.345) |
| 0.041 | 1.033 | 0.59 | 0.0046 | 0.147 |
| 0.039 | | 0.64 | 0.0055 | 0.188 |
| 0.053(0.045-0.067) | 1.899(1.835-2.000) | 0.88(0.76-1.07) | 0.0070(0.0064-0.0074) | 0.210(0.203-0.222) |
| 0.054(0.044-0.073) | 1.419(1.038-1.662) | 0.51(0.41-0.58) | 0.0041(0.0030-0.0048) | 0.180(0.161-0.207) |
| 0.042 | | 0.78 | 0.0059 | 0.203 |
| 0.061(0.034-0.095) | 2.092(1.748-2.504) | 0.92(0.64-1.17) | 0.0082(0.0070-0.0094) | 0.235(0.205-0.266) |
| 0.053(0.047-0.066) | 1.437(1.038-1.814) | 0.52(0.41-0.65) | 0.0043(0.0030-0.0054) | 0.179(0.168-0.190) |
| 0.045 | | 0.87 | 0.0062 | 0.211 |
| 0.071(0.029-0.129) | 2.346(1.690-2.567) | 1.12(0.89-1.74) | 0.0097(0.0076-0.0128) ³ | 0.261(0.241-0.328) |

| | | | | |
|--------------------|-----------------------|-----------------|-----------------------|--------------------|
| 0.104 | 3.698 | 1.35 | 0.0086 | 0.342 |
| 0.082 | | 2.07 | 0.0098 | 0.338 |
| 0.095 | 2.645 | 1.31 | 0.0161 | 0.374 |
| 0.201 | 6.708 | 2.20 | 0.0161 | 0.540 |
| 0.127 | | 3.27 | 0.0140 | 0.483 |
| 0.101 | 4.148 | 1.38 | 0.0251 | 0.616 |
| 0.137(0.123-0.152) | 4.863(4.004-5.722) | 2.29(1.87-2.72) | 0.0156(0.0134-0.0177) | 0.610(0.582-0.638) |
| 0.133 | | 3.41 | 0.0137 | 0.502 |
| 0.212(0.136-0.379) | 4.668(3.819-5.587) | 2.06(1.80-2.41) | 0.0204(0.0185-0.0248) | 0.494(0.424-0.553) |
| 0.343(0.307-0.379) | 8.030(7.047-8.413) | 3.30(2.82-3.79) | 0.0143(0.0130-0.0156) | 0.531(0.520-0.542) |
| 0.146 | | 3.71 | 0.0159 | 0.542 |
| 0.238(0.117-0.525) | 5.729(3.990-8.496) | 2.01(1.80-2.25) | 0.0238(0.0172-0.0394) | 0.583(0.416-0.920) |
| 0.337(0.188-0.486) | 8.599(7.756-9.442) | 4.04(3.94-4.13) | 0.0172(0.0156-0.0188) | 0.804(0.761-0.846) |
| 0.193 | | 4.70 | 0.0175 | 0.687 |
| 0.191(0.177-0.221) | 10.427(5.700-20.820) | 2.66(2.20-3.09) | 0.0154(0.0144-0.0160) | 0.676(0.646-0.733) |
| 0.295 | 8.107 | 2.95 | 0.0182 | 0.783 |
| 0.190 | | 4.63 | 0.0201 | 0.677 |
| 0.211(0.193-0.229) | 17.209(11.438-22.980) | 2.82(2.55-3.08) | 0.0205(0.0175-0.0236) | 0.659(0.659-0.660) |

TABLE 2—Continued

| TESTES | EPIDIDYMI | OVARIES | HYPOPHYSIS | PINEAL BODY |
|--------------------|-----------------------|-----------------------|-----------------------|------------------------|
| <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> | <i>grams</i> |
| 0.016(0.014-0.018) | 0.0078(0.0068-0.0095) | | 0.0012(0.0011-0.0014) | 0.00084(0.0007-0.0010) |
| 0.041 | | | 0.009 | |
| 0.046(0.039-0.054) | 0.015(0.012-0.020) | | 0.0016(0.0014-0.0019) | 0.00079(0.0006-0.0009) |
| | | 0.0018(0.0011-0.0026) | 0.0012(0.0011-0.0012) | 0.00077(0.0007-0.0008) |
| | | 0.0031 | 0.0009 | |
| | | 0.0033(0.0019-0.0047) | 0.0016(0.0010-0.0020) | 0.00076(0.0006-0.0010) |
| 0.042(0.033-0.051) | 0.015(0.013-0.016) | | 0.0016(0.0014-0.0018) | 0.00087(0.0008-0.0010) |
| 0.054 | | | 0.0011 | |
| 0.068(0.037-0.088) | 0.017(0.0100-0.0254) | | 0.0017(0.0015-0.0019) | 0.00073(0.0006-0.0008) |
| | | 0.0034 | 0.0016 | 0.0008 |
| | | 0.0039 | 0.0011 | |
| | | 0.0065(0.0056-0.0078) | 0.0018(0.0017-0.0019) | 0.0007(0.0006-0.0008) |
| 0.039(0.033-0.044) | 0.015(0.010-0.019) | | 0.0017(0.0014-0.0018) | 0.00079(0.0006-0.0009) |
| 0.060 | | | 0.0012 | |
| 0.059(0.045-0.070) | 0.014(0.012-0.015) | | 0.0021(0.0019-0.0022) | 0.00082(0.0006-0.0010) |
| | | 0.0028(0.0019-0.0040) | 0.0016(0.0013-0.0020) | 0.00069(0.0006-0.0008) |
| | | 0.0043 | 0.0012 | |
| | | 0.0043(0.0022-0.0062) | 0.0020(0.0017-0.0024) | 0.00084(0.0006-0.0010) |
| 0.139 | 0.026 | | 0.0022 | 0.0012 |
| 0.130 | | | 0.0018 | |
| 0.118 | 0.023 | | 0.0020 | 0.0012 |

| | | | | | |
|--------------------|--------------------|----------------------------|--|----------------------------|------------------------|
| | | 0.0108 0.0080 0.0203 | | 0.0030 0.0025 0.0030 | 0.0012 0.0010 |
| 0.394(0.378-0.411) | 0.059(0.055-0.064) | | | 0.0033(0.0031-0.0034) | 0.0012(0.0011-0.0013) |
| 0.321 | | | | 0.0026 | |
| 0.228(0.119-0.297) | 0.040(0.021-0.053) | | | 0.0028(0.0026-0.0030) | 0.0012(0.0011-0.0013) |
| | | | | 0.0031(0.0030-0.0031) | 0.0013(0.0013-0.0013) |
| | | 0.0090(0.0080-0.0100) | | 0.0028 | |
| | | 0.0085 | | 0.0034(0.0029-0.0041) | 0.0011(0.0010-0.0012) |
| | | 0.0105(0.0085-0.0128) | | | |
| 1.046(0.952-1.141) | 0.108(0.095-0.121) | | | 0.0042(0.0031-0.0052) | 0.0011(0.0010-0.0012) |
| 0.704 | | | | 0.0034 | |
| 1.053(0.934-1.099) | 0.315(0.257-0.428) | | | 0.0033(0.0028-0.0039) | 0.00075(0.0007-0.0008) |
| | | 0.0114 0.0095 | | 0.0036 0.0038 | 0.0008 |
| | | 0.0091(0.0072-0.0110) | | 0.0040(0.0034-0.0045) | 0.0008(0.0007-0.0009) |

¹ Average of 5 individuals.² Average of 6 individuals.³ Average of 3 individuals.⁴ Average of 2 individuals.⁵ Average of 1 individual.

* Pathological.

RATIO OF TAIL LENGTH TO BODY LENGTH

Immediately after death each rat was laid on its back and gently extended. The distance from the tip of the nose to the anus, and from the anus to the tip of the tail was carefully measured. From the measurements thus obtained individual ratios between tail length and body length were computed. The average of these tail ratios expressed as percentage of the nose-anus length is given in table 1 for each group of rats.

The data in table 1 show that with advancing age, the tail ratio increases in both the control and test rats. However, as compared with that in younger controls of corresponding body weight, the tail ratio, without exception, is much higher in the test rats. At ten weeks of age the ratio for the underfed individuals averages approximately 0.78 (73.9 per cent in the males and 82.0 per cent in the females), as compared with an average of 0.55 (53.7 per cent in the males and 56.6 per cent in the females) for the younger controls of corresponding weight. The differences at other ages, though somewhat less, are also very pronounced. It is therefore evident that in the underfed rats, the tail continues to grow in length more rapidly than the body. Thus the tail ratio in the underfed rats at ten weeks (0.78) approaches, but does not quite reach, the normal ratio of 0.88 (Jackson '15 b) for (larger) rats of corresponding age.

In the rats underfed for very long periods, however, the data (table 1) indicate an elongation of the tail (ratio 0.89-0.93) even beyond the normal ratio for corresponding age. A similar condition was observed by Jackson ('15 b) in two cases.

It may further be noted in table 1 that in the controls (except at one week of age), and also in the test rats, the tail in most instances averages relatively longer in the female than in the male. The longer tail in the female, as well as the marked elongation of the tail in young rats held at maintenance, is in agreement with the observations by Jackson ('15 b).

Hatai ('08) claimed that feeding rats upon an unsuitable diet of starch mixtures produced short-tailed individuals. Hatai ('15), however, found the tail length in five stunted rats fed upon

a lipid-free ration to exceed slightly the length given by the Wistar reference tables for normal rats of corresponding body length. Also in a later personal communication to Prof. C. M. Jackson he stated that in rats subjected to chronic inanition the tail becomes relatively long.

Harms ('09) observed that while starvation produced a marked decrease in the body length of Triton, the tail length remained practically unchanged. Morgulis ('11) however found the converse to be true in *Diemictylus*.

Jackson ('15 a) found that acute and chronic inanition in adult albino rats also tends to produce relatively long-tailed individuals, due probably to a shrinkage in the trunk length.

HEAD

In the test rats underfed from birth to three and ten weeks of age, the absolute weight of the head (table 1) is slightly higher than in the controls of corresponding body weight. In the test rats at three weeks of age there is an apparent increase from an average of 2.42 grams for the younger controls (sexes combined) to 2.83 grams, an increase of approximately 16 per cent (uncorrected for slight difference in body weight). The increase in head weight at this age is due in large part to the increase in the weight of the brain. At ten weeks of age, the increase of the head weight in the test rats (about 4 per cent) is very slight. At six weeks of age, the weight of the head for the underfed individuals (3.27 grams) is slightly lower than the average for the younger controls (3.40 grams). In the rats underfed for very long periods the weight of the head exceeds that in the controls 16 to 26 per cent.

In general, therefore, the data indicate that in very young rats underfed for considerable periods, the weight of the head slightly exceeds that of the younger controls of corresponding body weight, especially after very long periods of underfeeding.

Jackson ('15 b) similarly found in the majority of instances the head apparently to increase slightly in weight in young rats kept at maintenance for various periods. In adult rats subjected to both acute and chronic inanition Jackson ('15 a) found the

head to become relatively large, due to the very slight loss of the head in weight, as compared with the loss in weight of the body as a whole.

EXTREMITIES AND TRUNK

According to Jackson and Lowrey ('12) the upper extremities (fore limbs) at one week of age normally form an average of 8.92 per cent of the body, the average net body weight being 11.6 grams. In my controls of the same age, but somewhat lighter in weight (9.8 grams net), the average relative weight of the upper extremities (6.9 per cent) is lower. This difference may be due in part to variation in the technique used in removal of the extremities, though in both cases they were divided at the shoulder joint.

As compared with my controls the weight of the upper extremities (table 1) is heavier in the test rats underfed from birth to three weeks of age. The apparent growth from 0.678 to 0.800 gram (sexes combined) represents an increase of about 18 per cent. In the case of the rats underfed longer periods the weights of upper extremities are variable, but in the majority of instances they are lighter in the test animals than in the controls. On the whole, the differences are not very striking.

In the case of the lower extremities (table 1), the average relative weight of the hind limbs (9.53 per cent) for my controls at one week, is slightly lower than the relative weight (11.97 per cent) obtained by Jackson and Lowrey ('12) for normal rats of the same age.

As compared with my controls, the data in general indicate no distinct change in the weight of the hind limbs in the test rats. In the case of the four males underfed until 291 days of age there is an apparent increase from 11.58 grams in the controls to 14.20 grams in the test rats. The lower extremities are also much heavier in the females underfed to 314 days of age as compared with the younger control of corresponding body weight. At all other ages, however, the differences are variable and much less marked.

The weight of the trunk (table 1) is also variable, but in the majority of cases averages slightly lower in my test rats than in the controls. The differences, however, are small, and therefore, as in the case of the lower extremities, are of questionable significance.

On the whole, therefore, the data indicate that the weights of the extremities are practically normal in the test rats as compared with the younger controls of corresponding weight. The slight increase in the head, especially in the older groups, is apparently compensated for in most cases by a corresponding decrease in the weight of the trunk. Considerable allowance should be made, however, for experimental error and normal variability.

Jackson ('15 b) in somewhat similar experiments noted no distinct change in the body proportions. There was, however, an apparent slight increase in the head, counterbalanced by a corresponding decrease in the trunk and extremities. During inanition in adult rats, apparently both the head and the extremities increase in relative weight, whereas the trunk decreases (Jackson '15 a).

INTEGUMENT

In my young test rats underfed from birth to six and ten weeks of age, the weight of the integument (table 1) is considerably lower than in the controls. At 10 weeks of age the average decrease from 3.9 to 2.0 grams amounts to a loss of about 48 per cent. Jackson ('15 b) likewise observed that the integument suffers a considerable loss in weight in young rats held at maintenance for various periods. For my test rats underfed from birth to three weeks, however, the relative weight of the integument averages 21.13 per cent of the body weight, as compared with 21.05 per cent for the younger controls of corresponding body weight. Thus it is evident that in rats underfed from birth to three weeks there is practically no change in the weight of the integument, which is in marked contrast to the loss suffered in the test rats fasting for longer periods. The greater severity of the longer fasts as compared with the shorter period may account in part for the differences observed. This may be due to a

stronger growth tendency in the integument during the early maintenance period, or to a stronger tendency to accumulation of fat in the normal integument in the later periods.

In the test rats underfed for very long periods and weighing between 30 and 50 grams, the weight of the integument averages still somewhat lower than in the controls of corresponding weight, although the difference is less marked than in the ten weeks' group. The weight of the integument in the test rats weighing approximately 70 grams at the end of long periods of underfeeding, however, averages considerably higher than in the corresponding controls.

Aron ('11) found the skin in dogs held at constant weight to show a slightly higher percentage of the body weight than in normal control dogs. In these experiments, however, as pointed out by Jackson ('15 b), if comparison had been made with the controls at the beginning of the experiment instead of at the end, the opposite conclusion might have been reached. During acute and chronic inanition in adult rats, Jackson ('15 a) found the integument to lose weight in nearly the same proportion as the whole body.

Although the integument as a whole in most cases suffers a marked loss in weight, it was nevertheless observed that the external ear (fig. 1) in the young stunted rats, steadily continues to increase in size, and also changes its form so as to approach the normal adult appearance. The continued growth of the external ear is probably associated with the growth tendency of its skeletal (cartilaginous) portion.

SKELETON

The ligamentous, moist cartilaginous and dry cartilaginous skeletons were prepared as described by Jackson ('15 b). The moist skeleton was dried to constant weight in an oven at 85°C.

It is evident from the data in table 1 that the weight of the ligamentous skeleton is constantly higher in the test rats than in the controls of corresponding weight. In the test rats at ten weeks of age there is an apparent increase in the weight of the ligamentous skeleton from an average of 2.357 grams (sexes

combined) for the younger controls to 2.915 grams for the underfed individuals. The increase amounts to nearly 24 per cent (uncorrected for slight difference in body weight).

Similarly, the data for the moist cartilaginous skeleton (table 1) show a marked increase in the underfed rats. For the test ani-

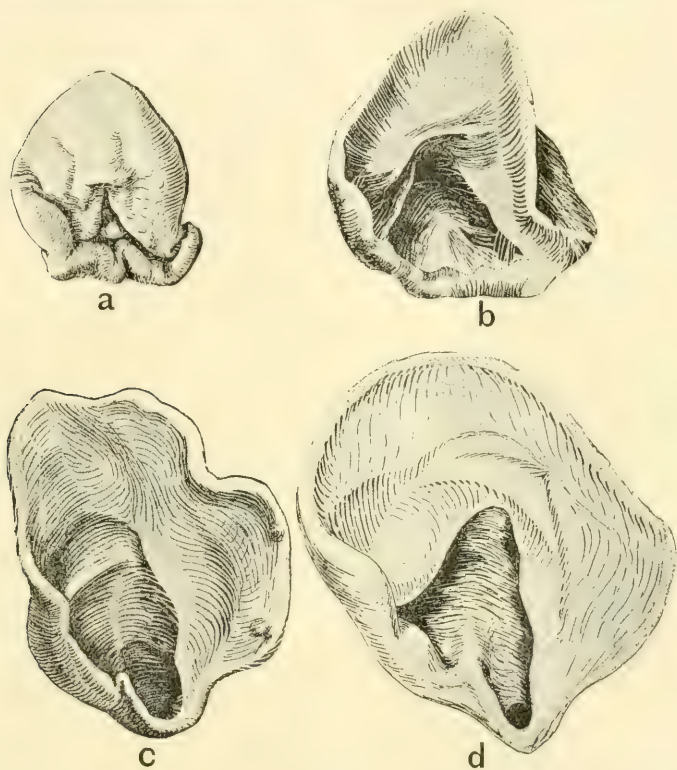


Fig. 1 a. External ear of a normal albino rat two weeks of age weighing 15.0 grams. $\times 4$. b. External ear of an albino rat underfed from birth to three weeks of age. Weight of rat was 11.5 grams. $\times 4$. c. External ear of an albino rat underfed from birth to ten weeks of age. Weight of rat was 15.4 grams. $\times 4$. d. External ear of a normal albino rat ten weeks of age weighing 89.0 grams. $\times 4$.

mals ten weeks of age the average absolute weight of the moist cartilaginous skeleton (2.470 grams) exceeds that for the younger controls (1.597 grams) by 0.873 gram, an excess of about 54 per cent. At other ages the differences are also very striking.

The weight of the ligaments and periosteum (obtained by subtracting the weight of the moist cartilaginous from the weight of the ligamentous skeleton) apparently has decreased considerably in the test rats at three, six and ten weeks of age. For the last group there is an apparent loss in weight from an average of 5.12 per cent of the body weight in the controls, to an average of 3.04 per cent in the test rats. The data for the ligaments and periosteum at the end of the very long fasting periods are variable, but in the majority of instances there is an indication of an increase in the weight of this portion of the skeleton.

Jackson ('15 b) in similar experiments starting at three weeks of age, found the ligamentous skeleton to manifest a marked growth tendency during maintenance.

The alkaline solution in which the skeletons are boiled probably acts more severely on the tender skeletons of my young controls than on the tougher and more calcified skeletons of the older test rats, thus giving an abnormally high apparent weight for the ligamentous portion of the skeleton in the controls. This would no doubt tend to mask any actual increase in the ligaments and periosteum in the rats underfed for the shorter periods. This may account for the difference between my results in earlier periods of fasting and those obtained by Jackson for the ligamentous structures.

In the case of the dried cartilaginous skeleton the data (table 1) indicate a greater percentage of dry substance in the skeletons of the test rats than in the controls. At ten weeks of age the percentage of dry substance amounts to 23.4 per cent of the moist cartilaginous skeleton in the controls and 30.3 per cent in the test rats. Also at other periods (both older and younger) the percentage of dry substance in the skeletons of the test rats exceeds that for the controls.

It is evident, therefore, that during the underfeeding the skeleton had gained in solids and lost in water content. Thus the continued growth of the skeleton had proceeded along the lines of normal development, for Lowrey ('13) finds the dry substance of the normal ligamentous skeleton to increase steadily

from an average of 33.3 per cent at twenty days of age to 52.6 per cent at one year.

Further evidence of growth continuing according to the normal process is indicated by the appearance of the third molar in the mandible and maxilla of the test rats underfed from birth to ten weeks of age. This tooth is not visible in the jaws of the younger controls with the same body weight.

The various individual bones of the skeleton in the underfed young rats are not only larger but also more advanced in their stage of development. The epiphyses at the ends of the humerus, especially at the distal end, are well fused with the shaft, much more so than in the case of the younger controls. Also the epiphyses at the ends of the femur and tibia are more completely developed in the test animals at ten weeks of age than in the controls, and in the former only has the tibia fused with the distal end of the fibula.

In general, therefore, the results confirm the observations of Jackson ('15 b) that during maintenance of body weight in young animals, not only skeletal increase in mass occurs, but also skeletal growth and differentiation of apparently normal character, though somewhat retarded in rate.

Waters ('08), who found that calves continue to increase in height and width of hip for a considerable time when held at maintenance, was probably the first to observe the fact that the skeleton continues to grow in young animals even when the body weight is held constant.

Aron ('11) noted an increase in the length and height of young dogs held at constant weight for considerable periods. He also found an increase in nine of the individual bones, but made no observations upon the entire skeleton. The first complete and systematic study of the growth of the skeleton in young animals held at maintenance was by Jackson ('15 b).

Birk ('11), Aron ('14), Hess ('16) and others show that a strong growth tendency of the skeleton is apparently manifested also in children during malnutrition.

During acute and chronic inanition in adult rats the skeleton nearly maintains its original weight (Jackson '15 a).

MUSCULATURE

The absolute weight of the musculature (table 1) averages constantly higher (except in the four test males at 412 days, and in one female at 316 days) in the underfed rats than in the controls of corresponding body weight. The apparent increase in the individuals underfed from birth to ten weeks from 3.85 grams to 4.24 grams (sexes combined), however, represents an increase of only 10 per cent. The greatest excess is in the four test males at 291 days, in which the weight of the musculature exceeds that of the (younger) controls by about 20 per cent. At other ages, however, the differences are not very striking. In the exceptional cases mentioned above the average weight of musculature for the test rats is slightly below that for the (younger) controls of the same body weight.

In general, therefore, it appears that the musculature in the test rats differs only slightly from that of the controls. The averages, however, indicate a slight increase in weight in the majority of cases. On account of the difficulty in removing the musculature completely in a uniform manner, the exceptions may be due to experimental error, rather than to variability in the musculature.

Jackson ('15 b) likewise observed but slight change in the weight of the musculature in young rats kept at constant body weight, although in the majority of cases there was a slight tendency to increase. Since the tendency is even greater and more constant in my series, it may be concluded with a fair degree of certainty that in young rats of various ages held at maintenance for various periods the musculature shows a slight but definite tendency to increase in weight in the great majority of cases.

During both acute and chronic inanition in adult rats, Jackson ('15 a) found the musculature to lose relatively in nearly the same proportion as the whole body, the loss being somewhat greater in chronic than in acute inanition.

VISCERA

The weight of the visceral group as a whole (table 1), including the abdominal and thoracic viscera, brain, spinal cord and eye-balls, in the rats underfed from birth to three, six and ten weeks of age, average constantly higher than in the corresponding controls. The increase in absolute weight amounts to approximately 28, 29 and 38 per cent in the test rats at three, six and ten weeks respectively. In the rats underfed from 21 to 412 days of age the increase is less marked, while in the test rats weighing 75 grams at 291 days there is an apparent decrease in the weight of the visceral group from 13.68 to 11.19 grams, a loss amounting to approximately 19 per cent.

From the foregoing, it is evident that in rats underfed from birth to three, six and ten weeks of age, there is a distinct increase in the weight of the visceral group. In the older test animals the changes are less marked, in some instances actually showing a considerable loss in weight. My data, however, indicate an increasing tendency to loss of weight in the visceral group in prolonged retardation of body growth, although the individual viscera differ in this respect, as will appear later.

Jackson ('15 b) likewise found the visceral group to show a distinct increase in weight in rats kept at maintenance from three to six, eight and ten weeks of age, but found no essential change in the later periods. According to Jackson ('15 a) the visceral group undergoes little change in relative weight in adult rats during acute and chronic inanition.

'REMAINDER'

The weight of the 'remainder' (table 1), which includes some small unweighed organs, fat, and body-fluids, was obtained by deducting the weight of the integument, skeleton, musculature and viscera from the net body weight. The intestinal contents are therefore not included.

In the rats underfed from birth to three weeks of age the 'remainder' has apparently suffered an average decrease from 1.843 to 1.092 grams or a loss of approximately 40 per cent, which

was compensated for by an increase in the skeleton and musculature. The marked decrease of the 'remainder' in these very young rats may possibly be due to a decrease in body fluids, the percentage of which is very high in young rats (Jackson and Lowrey '12), (Lowrey '13).

In the later periods the data show considerable variability in the 'remainder,' but in most instances the weight of the 'remainder' is higher in the later periods.

Jackson ('15 b) found considerable variability in the 'remainder' of young rats held at maintenance beginning at three weeks or later, but considered it doubtful whether there is any definite change in the test rats, as compared with the normal. In adult rats subjected to acute or chronic inanition, however, Jackson ('15 a) found a definite decrease in the weight of the 'remainder,' probably due to loss of fat.

BRAIN

The weight of the brain (table 2) in the test rats underfed from birth to three weeks of age greatly exceeds that in either the corresponding controls or the Wistar norm for rats of the same body length as the controls. There is an increase from an average (sexes combined) of 0.616 gram for my controls to 0.985 gram in the test rats. This is an absolute increase of 0.369 gram, or a relative increase of about 60 per cent (uncorrected for slight differences in body weight). The average relative weight (9.80 per cent) of the brain in this group of test rats greatly exceeds the normal maximum of about 6.7 per cent of the body weight observed by Jackson ('13) for rats weighing approximately 15 grams.

In the rats underfed for longer periods the weight of the brain is more nearly equal in test rats and controls. As shown in table 2, however, even in these groups the test rats (excepting the females at 42 days) slightly exceed the controls in absolute brain weight. The maximum apparent increase amounts to 13.3 per cent in the female at 316 days, and the average increase is about 5.8 per cent. This is subject to a small correction on account of slight differences in body weight. It may also be noted that the

brain in my controls is usually heavier than the Wistar norm for rats of corresponding body length.

From the foregoing it is evident that in rats underfed from birth to three weeks of age there is a marked increase in the weight of the brain, whereas during the longer fasting periods (both in those beginning at birth and those beginning at three weeks) the increase is very slight. Bechterew ('95) found acute inanition in puppies and kittens to produce an apparent loss of weight in all of the organs, including to a slight extent the central nervous system. The greatest loss suffered by the central nervous system was apparently in the cerebral hemispheres, whereas the smallest loss was in the spinal cord. Microscopic examination revealed evidence of delayed medullation in fiber tracts not fully developed when the fasting started.

Hatai ('04) noted an apparent absolute decrease of 5 per cent in the weight of the brain in young rats suffering a loss of approximately 30 per cent in body weight due to an unfavorable diet of starch and beef-fat.

Later, Hatai ('08) found the brain weight in rats stunted by feeding upon an unfavorable diet, to be practically identical with that for normal younger rats of the same body weight.

Donaldson ('11) found the weight of the brain in a large series of rats held nearly at maintenance from 30 to 51 days of age to average 7.7 per cent less than that for the full-fed controls at the same age. However, he points out that if comparison be made with the calculated initial brain weight, an increase of 3.6 per cent is apparent in the underfed rats. This in general agrees with the results of the present investigation.

Jackson ('15 b) observed practically no change in the weight of the brain in young rats held at maintenance for various periods starting at three weeks of age. My experiments started on much younger rats, and during a period when the brain normally shows a marked growth capacity. This probably accounts for the remarkable increase in brain weight observed for my youngest group. At later periods the brain loses its earlier intensity of growth, which is more nearly equalled by the remainder of the body. During acute and chronic inanition in adult rats the brain loses little if any in absolute weight (Jackson-'15 a).

SPINAL CORD

The weight of the spinal cord (table 2) like the brain in my controls averages slightly higher than the Wistar norm for rats of corresponding length, the only exceptions being the two control males and one control female weighing about 70 grams net. The differences are less marked when the body weight is used as a basis for comparison. As compared either with my controls or with the Wistar tables, the spinal cord is constantly heavier in the test rats. In the test rats at three and ten weeks of age, the average increase (sexes combined) amounts to approximately 70 per cent, and at six weeks to about 38 per cent. In the other groups of test rats underfed for long periods, the increase while still considerable, is somewhat less marked, varying from 29 to 56 per cent in the various groups (table 2).

Donaldson ('11) also found an increase in the weight of the spinal cord in rats held at 34 grams body weight from 30 to 51 days of age.

Jackson ('15 b) found that during maintenance there was a well-marked increase in the weight of the spinal cord, amounting to 36 per cent in those at maintenance from three to ten weeks of age.

It therefore appears that in young rats underfed at various ages the spinal cord shows a remarkable tendency to grow, this tendency being relatively strongest in those stunted from birth. This increase in weight is also maintained (to a lesser degree) in young rats underfed through long periods of time.

According to Jackson ('15 a) the spinal cord suffers practically no loss in absolute weight during acute and chronic inanition in adult rats.

EYEBALLS

The weight of the eyeballs in my controls (table 2) averages constantly higher than the Wistar norm for rats of corresponding length. The differences, however, are not striking and are probably due largely to differences in body weight (my rats averaging slightly heavier than the Wistar norm for corresponding length).

When the weight of the eyeballs for the controls is compared with the weight for the test rats, it is evident that a marked increase has occurred in the latter. At ten weeks of age the increase in weight from an average of 0.093 gram in the controls to 0.155 gram in the test rats, equals an increase of more than 66 per cent. At six and ten weeks of age the excess weight in favor of the test rats is less, being about 41 and 34 per cent respectively. The increase at later periods, in some instances is even more striking, amounting to approximately 73 per cent (sexes combined) in the rats weighing nearly 50 grams after very long periods of fasting.

My results, therefore, confirm Jackson's ('15 b) observation that the eyeballs show a marked growth capacity in young rats kept at maintenance for various periods. He suggests that the possibility of continued growth of the eyeballs is due largely to water absorption, the water content of the eyeballs being normally very high (85.6 per cent at 20 days according to Lowrey' 13).

The eyeballs suffer practically no loss in weight during acute and chronic inanition in adult rats according to Jackson ('15 a).

A few observations were made concerning the time at which the eyelids opened in my albino rats. The data (table 3) indicate that the eyelids opened in my controls at an average age of fifteen days, whereas in the test rats the opening is apparently delayed until about the seventeenth day. The body weight of the controls when the eyelids opened averaged 16.5 grams as compared with 9.9 grams for the test rats. Therefore, although somewhat retarded in time, the eyelids nevertheless opened at a lighter body weight in the underfed individuals than in the controls. However, in the case of one litter (St 63) it was noted that the eyelids opened in two test rats weighing an average of 12.2 grams even at a younger age (15 days) than in the control. The exact age at which the eyes opened in the controls in this instance was not recorded except that it occurred after the fifteenth day at a body weight of more than 15.5 grams.

Bechterew ('95) likewise found a delay in the opening of the eyelids in newborn dogs and cats subjected to acute inanition.

Several investigators have noted the age at which the eyes

normally open in the albino rat. Jackson ('12) states that the eyes are opened at about sixteen or seventeen days of age. Donaldson ('15) ('The Rat,' p. 19) states that the eyes open from the fourteenth to the seventeenth days, most often on the fifteenth or sixteenth.

King ('16) states that usually the female rats in a litter open their eyes several hours sooner than do the males. The delay in the opening of the eyelids observed in my test rats apparently is not because the data, include a large majority of females, for

TABLE 3
*Age and gross body weight at which the eyelids opened in the control and tests rats**

| CONTROLS | | | | TEST RATS | | |
|---------------|-------------------------------|-------------|-------------------|-------------------------------|-------------|-------------------|
| Litter number | Number of individuals and sex | Age in days | Gross body weight | Number of individuals and sex | Age in days | Gross body weight |
| | | | grams | | | grams |
| St 56 | 1(F) | 15 | 16.0 | 3(1 M, 2 F) | 18 | 7.9 |
| St 59 | 1(F) | 15 | 11.3 | 8(1 M, 7 F) | 18 | 8.6 |
| St 63 | 1(F) | 15+ | 15.5+ | 2(F) | 15 | 12.2 |
| St 68 | 1(M) | 17 | 20.0 | 1(F) | 18 | 14.0 |
| St 92 | 2(1 M, 1 F) | 15 | 20.0 | 2(1 M, 1 F) | 16 | 11.0 |
| St 95 | | | | 1(M) | 18 | 11.0 |
| St 102 | 1(F) | 14 | 15.0 | 6(F) | 17 | 10.2 |
| St 106 | 1(F) | 14 | 15.0 | 2(1 M, 1 F) | 19 | 10.5 |
| St 114 | 2(1 M, 1 F) | 15 | 15.8 | | | |
| Average* | 10(3 M, 7 F) | 15 | 16.5 | 25(5 M, 20 F) | 17.5 | 9.9 |

* Control of Litter St 63 excluded from the averages, as the exact time at which the eyelids opened was not recorded.

in practically every instance (see exception noted) the eyelids opened two or three days later in the test females than in the female controls of the same litter.

THYROID GLAND

In the test rats fasting from birth to three, six and ten weeks of age the weight of the thyroid gland (table 2) is practically identical with that in the controls. However, in the rats underfed for very long periods, in which the experiments started at three weeks of age, the weight of the thyroid in most instances

shows a marked decrease in the test rats when compared with either my controls or with the Wistar norm tables. For example, in the case of the eight test rats weighing about 50 grams after very long underfeeding the thyroid has apparently suffered a loss of approximately 50 per cent in the males, although there is apparently no change in the females.

These latter results therefore agree in general with those obtained by Jackson ('15 b) in rats held at maintenance from the age of three to six, eight and ten weeks. In my younger rats, however, subjected to inanition for various periods starting at birth, the weight of the thyroid apparently remains practically unchanged. The power of maintenance in the thyroid therefore appears stronger in the very young rats. Considerable allowance should be made for experimental error due to the difficulty in dissecting the thyroid gland in an accurate manner.

During acute inanition in adult rats, Jackson ('15 a) reports that the thyroid gland apparently loses little or no weight, while in chronic inanition with an average loss in body weight of 36 per cent, the thyroid gland loses only about 22 per cent in weight.

THYMUS

As is evident from table 2, the weight of the thymus is, without exception, much lighter in the test rats than in the controls. At three, six and ten weeks of age the loss in weight amounts to approximately 30, 60 and 80 per cent respectively. The decrease in weight of the thymus in the test rats, while very marked in all groups, is especially striking in the four test males underfed 412 days. From an average of 0.207 gram in the controls, the weight in the test rats has decreased to 0.011 gram, a loss of approximately 94 per cent. This decrease is not so remarkable, however, when the normal involution with age is taken into account. According to the Wistar tables, the normal weight of the thymus at 400 days is only 0.039 gram.

Thus it is evident, as was observed by Jackson ('15 b) that the thymus loses markedly in weight in young rats underfed for various periods. He found a loss of about 90 per cent for the

thymus in rats held at maintenance from the age of three to ten weeks.

Jonson ('09) likewise found in young rabbits kept at constant body-weight for four weeks the weight of the thymus to be reduced to about one-thirtieth its initial value. The greatest loss is suffered by the cortex, which is reduced to one-twelfth of its initial weight during two weeks of maintenance. Judging from comparison with my own results, as well as with those of Jackson, the process of hunger involution of the thymus would appear to be much more rapid and complete in the rabbit than in the rat.

HEART

The weight of the heart (table 2) in the rats underfed from birth to three weeks of age differs but little from that in the controls (decrease of about 10 per cent in the males; slight increase in the females). At six and ten weeks of age, however, there is an evident increase in the heart weight. In those underfed from birth to six weeks, the average increase is about 13 per cent. For the groups underfed to ten weeks, the increase from an average of 0.092 gram in the controls to 0.117 gram in the test rats (sexes combined) represents an increase of about 27 per cent (subject to slight correction for differences in body weight). In the rats underfed for very long periods, the heart weight, while variable in most instances, is lower than in the controls. There is a decrease in the various groups, varying from about 8 to 29 per cent, excepting the four females at 392 days, which show an apparent increase of about 3 per cent (most of which may be accounted for as due to difference in body weight).

In general, therefore, the data indicate a slight increase in the weight of the heart in the test rats at six and ten weeks of age, while during the later periods the heart apparently lost weight in the majority of instances. However, on account of the normal very considerable variability of the heart weight, especially in young rats (Jackson '13), the apparent changes in the heart during underfeeding are probably somewhat doubtful.

In young rats kept at constant body weight for various periods,

and also in adult rats subjected to acute and chronic inanition Jackson ('15 a, '15 b) found the percentage weight of the heart to remain practically unchanged. Apparently the effects were less marked than in my experiments, in which the underfeeding was begun earlier, or carried over longer periods.

LUNGS

As compared with the Wistar norm for rats of corresponding body length, the weight of the lungs (table 2) appears to be unusually high in my young controls. For the largest group (14 rats, 7 males and 7 females, 13 days old) weighing about 15 grams, the average absolute weight of the lungs (sexes combined) is 0.234 gram, as compared with the Wistar average of 0.166 gram for rats of corresponding body length.

Jackson ('13) found the normal weight of the lungs in the rat at one week to average 0.1938 gram, with which my average of 0.1995 gram for controls of the same age agrees closely. It appears, therefore, as pointed out by Jackson ('13), that weights for the lungs derived from Hatai's formula appear much lower than the actual observed values in very young rats.

As compared with my controls, the average weight of the lungs is low in the test rats at three, six and ten weeks of age, the decrease in each case amounting to a loss of approximately 26 per cent (subject to slight correction for difference in body weight).

In the longer experiments the lungs average heavier for the underfed rats than in the controls, with the exception of one female at 139 days. The presence of lung infection among the older underfed rats probably accounts in part at least for the increase in the weight of the lungs. The data for the test rats weighing about 70 grams after very long fasting also include the weights of the lungs for five rats which were asphyxiated. In these instances the weights would no doubt be high due to the greater amount of blood retained in the lungs. Therefore the effect of the very long fasting period upon the weight of the lungs is doubtful.

Jackson ('15 b) found that in young rats subjected to maintenance for various periods, the lungs show a slight decrease in

weight during the earlier periods (decrease of 15 per cent) but not later. It is evident from my results that the loss in the lung weight in very young underfed rats is considerably greater.

During acute and chronic inanition in adult rats the lungs lose weight in about the same proportion as the whole body (Jackson '15 a).

LIVER

The weight of the liver (table 2) in my young control rats is very much lower than the Wistar norm for rats of corresponding body length. The differences are especially marked in the controls weighing approximately 13 and 15 grams. My average value of 0.37 gram at one week, however, corresponds rather closely with the weight of 0.3431 gram obtained by Jackson ('13) in rats of the same age and weight. Thus, as pointed out by Jackson, it seems that weights for the liver obtained by Hatai's formula, especially for young rats, are much too high, at least for rats of the Missouri and Minnesota colonies. The difference appears too great to be attributed to normal variability or to slight differences in diet. However, C. Watson ('10) finds a marked decrease in the relative size of the liver in captured wild rats fed upon bread and milk diet used during captivity; and Jackson ('13) has emphasized the marked and irregular variability found in the liver of the rat.

In the rats underfed from birth to three, six and ten weeks of age the weight of the liver is considerably higher than in the corresponding controls. The increase at three weeks averages about 17 per cent. The difference is especially marked, however, at six and ten weeks, amounting to an average increase of about 83 per cent in the former and 64 per cent in the latter (subject to correction for slight differences in body weight).

In the rats underfed for longer periods the liver is variable. At 412 days of age the average weight of the liver (2.04 grams) in four test males is practically identical with that in the two controls (2.06 grams). The liver weight of the controls appears to be abnormally low, however. In the remaining groups of test rats underfed for very long periods the weight of the liver is con-

siderably lower than in the corresponding controls. At 291 days of age there is an apparent decrease in the weight of the liver from 4.19 grams to 2.56 grams, a loss of about 39 per cent (uncorrected for slight difference in body weight).

In general therefore the data indicate that in young rats underfed for various periods, the liver shows a marked growth tendency in the very young rats, while in the very long fasting experiments there is usually a marked decrease in the weight of the liver.

Jackson ('15 b) likewise found that in young rats (three weeks old or more) held at maintenance the liver increases slightly (about 10 per cent) in weight during the shorter fasting periods; but in experiments extending over longer periods there is a decided decrease in the liver weight.

During inanition in adult rats, the liver loses in weight relatively more than the whole body, and to a greater extent in acute than in chronic inanition (Jackson '15 a).

SPLEEN

From table 2 it is very evident that in my young controls weighing approximately 10, 13 and 15 grams, the weight of the spleen greatly exceeds the Wistar norm for the spleen in rats of corresponding body-length. This is especially true for the youngest group. However, my average weight for the spleen (0.051 g.) at one week agrees more nearly with Jackson's ('13) normal spleen weight (0.0425 g.) at the same age. It has been noted by Jackson ('13) that the weight of the spleen derived from Hatai's formula is in general somewhat too low, probably because Hatai (without apparent justification) excluded all 'enlarged' spleens from his data.

The weight of the spleen (table 2) at three weeks of age is considerably lower in my test rats than in the controls. In this group there has been an apparent decrease from 0.051 to 0.026 gram, a loss of about 49 per cent (uncorrected for slight difference in body weight).

In the rats underfed from birth for longer periods (up to six

and ten weeks), however, the spleen shows a marked tendency to growth, the average apparent increase being about 27 per cent.

In rats underfed for longer periods, beginning at three weeks of age, on the contrary, there is an apparent decrease in the weight of the spleen, except in the four females at 412 days. On account of the extreme normal variability of the spleen, however, the apparent atrophy during the later fasting periods may be of doubtful significance. It is nevertheless in agreement with the doctrine that lymphoid tissues in general tend to atrophy during inanition (cf. Jolly et Levin, '11).

Jackson ('15 b) noted a reduction in the weight of the spleen in young rats during the earlier periods of underfeeding (beginning at three weeks), while at later periods there was no material change in weight. In adult rats during chronic inanition the average loss in weight is nearly proportional to that of the entire body while in acute inanition the loss appears very much greater (Jackson '15 a).

• STOMACH AND INTESTINES

The average weight of the stomach and intestines (including contents) is 1.10 grams (table 2) for my controls at one week. This is somewhat higher than the normal weight 0.733 gram obtained by Jackson ('13) for a large series of the same age. The difference is not entirely due to a difference in the amount of the contents, for the weight of the empty alimentary canal (0.36 gram) likewise averages higher than Jackson's weight (0.296 gram).

The weight of the contents of the alimentary canal (obtained by deducting the weight of the empty from the full alimentary canal) is much lower at three weeks in the test rats than in the controls. At six and ten weeks of age, however, the weight of the contents is excessively heavy in the underfed rats. In the later periods of underfeeding the data are variable but show a tendency to decrease.

Jackson ('15 b) noted an increase in the contents of the alimentary canal during the early periods of underfeeding, while during the longer experiments the change (a tendency to decrease) was

less marked. This is in agreement with my findings, excepting the youngest group.

As compared with my controls, the weight of the empty alimentary canal is higher in the test rats at three, six and ten weeks of age than in the controls. At ten weeks of age the weight of the empty digestive canal apparently has nearly doubled in the underfed animals.

At the later periods, however, the data indicate a reduction in weight of the empty alimentary canal, which is especially striking (34 per cent decrease) in the 4 males weighing about 75 grams at 291 days of age.

Jackson ('15 b) likewise found the empty alimentary canal to increase in weight during the shorter maintenance periods, after which there was a decline in weight.

During both acute and chronic inanition in adult rats there is a very marked decrease in the weight of the stomach and intestines, both with and without contents (Jackson '15 a.).

SUPRARENAL GLANDS

The average weight of the suprarenals (table 2, sexes combined) (0.0027 g.) for my controls at one week agrees fairly with the normal average (0.00226 g.) obtained by Jackson ('13), but is far below the weight given in the Wistar tables for rats of corresponding body length. Thus the weights for the suprarenals at one week derived from Hatai's formula seem much too high, at least for normal rats from the Missouri and Minnesota colonies. For the controls at 13 and 14 days of age (table 2) the difference is less marked.

As compared with my controls, the weight of the suprarenals (except in the four males at 291 days) is constantly higher in the test rats. The average increase in the weight of the suprarenals (sexes combined) in the test rats at three and six weeks of age amounts to more than 60 per cent (uncorrected for slight difference in body weight). At ten weeks of age the difference has reached a maximum average of approximately 114 per cent. At later periods (except in the four males at 291 days, in which

instance there was a loss of about 11 per cent) the suprarenals in the test rats exceed those of the corresponding controls 12 to 87 per cent. It should be noted that the weight of the suprarenals is slightly higher in the test females than in the males of corresponding body weight at ten weeks of age and also at all subsequent periods, thus indicating the appearance of sexual differentiation during underfeeding.

Jackson ('15 b) also found the suprarenals to manifest a marked growth tendency in young rats held at maintenance. He noted a maximum increase of 39 per cent in the female underfed from three to ten weeks of age. Apparently the growth of the suprarenals was less marked than in my experiments, in which the underfeeding was begun earlier or carried over longer periods. As occurs normally, the suprarenals during underfeeding underwent sexual differentiation in weight, also confirming the results of Jackson. In adult rats, there is but little loss in the absolute weight of the suprarenal glands during inanition (Jackson '15 a).

KIDNEYS

Using my controls as a basis for comparison, there is apparently an increase in the weight of the kidneys (table 2) in the rats underfed from birth to three, six and ten weeks of age. The apparent average increase (uncorrected for difference in body weight) amounts to about 21 per cent at three weeks, 45 per cent at six weeks, and 38 per cent at ten weeks.

In the rats underfed for very long periods the weight of the kidney is variable, some showing an increase and others a decrease. Due to this inconstancy in results, the changes are of questionable significance.

In general therefore it appears that, as was observed by Jackson ('15 b) the kidneys tend to increase in weight during the earlier periods of the experiment, but show no definite change during later periods. The increase in my young rats is more striking than the increase observed by Jackson. This is probably due to the fact that my rats were placed upon the experiment at a younger age, when the intensity of growth in the kidney is greater.

In adult rats during acute and chronic inanition Jackson ('15a)

found the kidneys to lose weight relatively slightly less than the entire body.

TESTES AND EPIDIDYMIDES

From the data in table 2 it can be seen that the Wistar data for the testes greatly exceed the corresponding weights for my control rats at 7 and 13 days of age. At one week the difference (0.016 to 0.041 gram) is especially striking. However the combined average weight of the testes and epididymides (0.0238 g.) for my controls at one week corresponds closely with Jackson's ('13) normal (0.0273 g.) at the same age. It is therefore apparent that the Wistar data are much too high for these young rats. For the older controls, my data correspond fairly well with the Wistar tables.

Using my controls for comparison the weight of the testes in the test rats is exceedingly high at three, six and ten weeks of age. At three weeks the weight of the testes in the test rats exceeds that for the younger controls of the same body weight approximately 188 per cent. At six and ten weeks of age the increase (uncorrected for differences in body weight) amounts to about 62 and 51 per cent respectively, having become progressively less.

In the rats weighing 75 grams at 291 days the weight of the testes is practically identical with that for the controls. At 139 and 412 days of age, however, the testes have evidently suffered a marked loss in weight. The decrease (0.394 gram to 0.228 gram) in the test males of the latter group amounts to a loss of about 42 per cent.

From the foregoing it is evident that in young rats the testes increase considerably during the shorter periods of underfeeding. With very prolonged stunting, however, the testes may show practically no change, or a loss which varies up to a considerable amount. Jackson ('15a) noted an increase of 34 per cent in the weight of the testes in young rats held at constant body weight from three to ten weeks of age. My results are much more striking, however, which is probably due to the stronger growth tendency of the testes in my very young rats which were underfed from birth. Considerable caution must be exercised in drawing

conclusions, however, on account of physiological variability in the weight of the testes.

The weight of epididymides (table 2) in my test rats of three and six weeks of age is higher than for the corresponding controls. For the youngest group there is an apparent increase from a normal weight of 0.0078 gram to 0.0154 gram an increase of over 95 per cent (uncorrected for slight difference in body weight). At six weeks of age the excess weight for the epididymides in the test rats is only 13 per cent.

In the rats underfed to ten weeks of age and also in those at 412 days, there is an apparent loss in the weight of the epididymi. The loss at ten weeks amounts to approximately 6 per cent, and at 412 days to about 32 per cent. However, in the test rats weighing 67.2 grams net at 291 days there is an apparent increase in the weight of the epididymi from 0.108 to 0.315 gram. In all cases the percentage losses above estimated are subject to correction for slight difference in body weight.

In general, therefore, the data indicate that in the youngest rats there is a marked increase in the weight of the epididymides, but that later there is a loss of weight in most cases.

Jackson ('15 b) found an apparent slight loss in the epididymides in rats held at constant body weight from three to six and ten weeks of age. In adult rats during inanition Jackson ('15 a) found that the testes and epididymides apparently lose weight in about the same proportion as the entire body.

OVARIES

The weight of the ovaries (table 2) in my controls at seven days of age (0.0018 g.) is very much lower than the Wistar norm (0.0033 g.) for corresponding body length. The difference amounts to more than 80 per cent. Jackson ('13) found the weight of the ovary to average 0.00121 g. at one week of age, which is even lower than my data. It therefore appears fairly certain that the weight of the ovary calculated from Hatai's formula is too high for this early period. At later ages my data do not differ so greatly from the Wistar tables.

In my rats underfed from birth to three, six and ten weeks of age, there is apparently a considerable increase in the weight of the ovary. At ten weeks the increase from an average of 0.0028 gram in the controls to 0.0043 gram in the test rats represents a gain of approximately 54 per cent (uncorrected for body weight). At three and six weeks of age the differences are greater, amounting to 83 and 91 per cent respectively.

The data for the ovaries in the females subjected to very long periods of fasting are variable, but in most instances show a tendency to increase. Conclusions, however, are hazardous on account of the small number of observations and also on account of the extremely great cyclic changes in the weight of the ovary due to ovulation. Also the normal growth curve of the ovary is very complicated, as shown by Hatai ('13).

Jackson ('15 b) observed in the ovaries a decrease of about 27 per cent in weight in young rats kept at constant body weight from three to ten weeks of age. His data, however, indicate a slight increase in the youngest group underfed from three to six weeks. My results would indicate that at earlier periods the growth impulse of the ovary in underfed rats is still stronger, resulting in a marked increase in its weight.

HYPOPHYSIS

The weight of the hypophysis (table 2) appears higher in my controls (except for the one female weighing about 70 grams) than the Wistar norm for rats of corresponding body length. The high weight for the gland in my controls can be attributed partly to differences in body weight, my rats being slightly heavier than the norm for corresponding body length.

As compared with my controls, the weight of the hypophysis at three, six and ten weeks of age is higher in the test rats than in the controls. At three and ten weeks of age the increase (sexes combined) for the test rats amounts to approximately 33 and 24 per cent respectively (uncorrected for slight difference in body weight). At six weeks of age the increase is less, amounting to about 8 per cent. There is apparently no sexual difference in the increase at these ages.

In the test rats underfed for very long periods, however, the hypophysis is variable. In the test males at 412 days there is an apparent average decrease in absolute weight from 0.0033 gram to 0.0028 gram, a loss of over 15 per cent. The decrease in the males at 291 days of age amounts to approximately 21 per cent. On the other hand, in the females fasting for long periods there is no such decrease. In fact for the one female at 316 days the weight of the hypophysis shows no change, whereas at 392 and 314 days there is an apparent average increase of approximately 10 and 11 per cent respectively (subject to slight correction for difference in body weight). It should be noted that the weight of the hypophysis is slightly higher in the very old test females than in the males, suggesting that sexual differentiation in weight has occurred.

In general, therefore, it appears that the hypophysis increases considerably in weight in underfed rats during the earlier periods. In the very long experiments however the sexes appear to react differently. The weight of the hypophysis in the males apparently suffers a loss during very prolonged fasting, but in the females it shows a slight increase in weight. Jackson ('15 b) similarly noted a distinct tendency to an increase with the appearance of sexual differentiation in the weight of the hypophysis in rats held at constant weight from three to ten weeks of age. During inanition in adult rats the relative weight of the hypophysis is practically unchanged (Jackson '15 a).

Quite recently Jackson ('17) has studied the volume-changes in the lobes of the hypophysis in rats underfed for various periods. In young rats kept at constant body weight the pars anterior is somewhat reduced in relative size, the intermedia and nervosa becoming correspondingly larger.

PINEAL BODY

The weight of the pineal body (table 2) is practically normal in each group of test rats when compared with the corresponding controls. The slight differences which exist (especially in some of the older groups) are of doubtful significance, especially when the exceedingly small size of the gland is taken into consideration.

Considerable allowance must be made for experimental error, and for variability (the extent of which is unknown).

The data for the control rats indicate no sexual difference in the weight of the gland at the ages observed. Hoskins ('16) likewise found no significant difference according to sex.

DISCUSSION

In general, the results of the present investigation concerning the effects of underfeeding upon very young rats and upon slightly older rats underfed for very long periods agree fairly well with those obtained by Jackson ('15 b) in rats held at maintenance for various periods beginning at three weeks of age. There are, however, certain differences found in my rats in which the underfeeding began shortly after birth, or was prolonged for very considerable periods. These differences are probably due chiefly to the varying tendencies to growth and maintenance among the various organs at different periods.

With reference to their growth tendency in young rats held at constant body weight, the organs are divided by Jackson ('15 b) into three classes: (1) those having a growth tendency so strong that they continue to increase, even when the body weight is held constant; (2) those which approximately hold their weight constant (variation within 10 per cent) under these conditions; and (3) those which are unable to maintain themselves and lose in weight.

The distribution of the organs according to this scheme for my rats underfed from birth to ten weeks, also for those weighing about 50 grams after very long periods of fasting, together with Jackson's ('15 b) results for rats underfed from three to ten weeks are shown in table 4.

On comparing my results with Jackson's it appears that in very young rats underfed from birth to ten weeks of age a larger number of organs show a tendency to increase than in rats underfed from three to ten weeks. On the other hand, very long fasting apparently adds to the number of organs which fall into the group showing approximate maintenance or a tendency toward loss in weight.

TABLE 4

Comparison of growth tendency; (1) in young rats underfed from birth to ten weeks; (2) in young rats held at maintenance from three to ten weeks; and (3) in rats weighing approximately 50 grams after very long fasting

| | MARKED TENDENCY TO GROWTH DURING UNDERFEEDING | PER CENT OF CHANGE | WEIGHT NEARLY CONSTANT DURING UNDERFEEDING | PER CENT OF CHANGE | MARKED LOSS IN WEIGHT DURING UNDERFEEDING | PER CENT OF CHANGE |
|--|---|--------------------------|--|-----------------------|---|-----------------------|
| Rats underfed from birth to ten weeks of age (Stewart) | Suprarenals | +114 | Musculature | +10 | Thymus | -80 |
| | Alimentary canal | +100 | Brain | +8 | Integument | -48 |
| | Spinal cord | +70 | Thyroid | +4 | Lungs | -26 |
| | Eyeballs | +66 | Epididymi | -6 | | |
| | Liver | +64 | | | | |
| | Ovaries | +54 | | | | |
| | Testes | +51 | | | | |
| | Kidneys | +38 | | | | |
| | Heart | +27 | | | | |
| | Spleen | +24 | | | | |
| Rats held at constant body weight from three to ten weeks of age (Jackson) | Hypophysis | +24 | | | | |
| | Skeleton | +24 | | | | |
| | Eyeballs | +50 | Liver | +10.3 | Thymus | -90 |
| | Spinal cord | +36 | Kidneys | +4.1 | Spleen | -42 |
| | Testes | +34 | Musculature | +3.0 | Integument | -36 |
| | Skeleton | +28 | Brain | -0.5 | Ovaries | -27 |
| | Alimentary canal | +28 | Heart | -0.6 | Thyroid | -24 |
| | | | | | Lungs | -15 |
| | Suprarenals | +12 +39 +18 +19 | | | | |
| | Hypophysis | | | | | |
| Rats weighing about 50 grams after very long periods of fasting (twenty-one to about four hundred days) (Stewart) | Eyeballs | +73 | Lungs | +28(?) | Thymus | -90 |
| | Spinal cord | +40 | Hypophysis, F | +10 | Testes | -42 |
| | Suprarenals | +31 | Brain | +4 | Epididymi | -32 |
| | | +66 | Musculature | +4 | Alimentary canal | -27 |
| | Skeleton | +32 | Spleen | -5 | | |
| | Ovaries | +17 | Kidneys | -6 | Thyroid | -36 |
| | | | Liver | -7 | Hypophysis | -15 |
| | | | Heart | -10 | Integument | -11 |

Thus the liver, ovaries, kidneys, heart and spleen show a marked tendency to increase in weight in the rats underfed from birth to ten weeks, but not in those underfed from three to ten weeks. On the other hand, in my rats fasting for very long periods, the hypophysis (in the males), alimentary canal, testes and epididymi show a marked loss in weight, which does not occur in rats underfed for shorter periods.

The present experiments thus emphasize the fact that with advancing age, many of the different organs show a changing tendency in their growth reaction during underfeeding. The same organ (for example, the heart or alimentary canal) in young animals during the earlier periods of fasting may manifest a marked tendency toward continued growth while the increase in body weight is greatly retarded, but later may barely maintain itself, and finally perhaps even lose weight. Other organs, like the eyeballs, spinal cord and skeleton, continue to grow or maintain their weight with remarkable persistency; while still others (thymus) show a marked loss throughout all periods of inanition. These changes in weight in the various systems and organs of the young body during inanition are probably correlated with histological, chemical and physiological changes concerning which as yet but little is known.

SUMMARY

The more important results of the present investigation may be summarized briefly as follows:

Newborn albino rats are able to withstand separation from the mother for nearly one-half of the total time for the usual nursing period of three weeks, resulting in great retardation of the normal growth of the body as a whole.

During the underfeeding, the tail in the test rats elongates more rapidly than the body, thus producing relatively long-tailed individuals.

The eyelids in the test rats open at a body weight lower than in the controls, but the time of opening is somewhat delayed.

As to the body proportions, the weights of the extremities are

but slightly (if at all) changed in the test rats during both short and very long fasting periods. The head apparently increases slightly in weight, especially during the very long fasts, the increase being compensated by a slight decrease in the weight of the trunk.

The results for the various systems are as follows:

The skeleton shows a considerable increase in weight in the test rats. The growth apparently proceeds along the lines of normal development, as indicated by a decrease in water-content, the appearance of third molar teeth, and by formation and fusion of various epiphyses.

The musculature in the test rats increases slightly in weight in the majority of cases.

The visceral group (as a whole) shows a considerable increase in weight in the test rats underfed from birth to three, six and ten weeks of age. During the longer fasts there is apparently a less definite change, with a tendency to decrease in the majority of cases.

The integument remains practically normal in weight in the rats underfed from birth to three weeks, but shows a marked loss in weight later. Although the integument as a whole loses weight during underfeeding to ten weeks of age, nevertheless the external ear continues to grow considerably in size, and tends to assume the normal adult appearance. The continued growth of the ear is probably associated with the persistent growth tendency of its skeletal (cartilaginous) portion.

The 'remainder' appears to decrease in the rats underfed to three weeks of age, which is partly compensated for by skeletal increase. During the longer fasts there is a slight increase in the weight of the 'remainder' in the majority of cases.

In general, therefore, in young rats subjected to prolonged inanition, there appears to be a progressive tendency in the skeleton and (to a slight extent) in the musculature to increase in weight, counterbalanced by a decrease in the integument and viscera. The individual organs of the visceral group vary greatly, however.

The brain shows a very marked increase in weight in the test

rats underfed to three weeks, and also to a slight extent at later periods.

The spinal cord shows a marked increase in weight in the test rats up to ten weeks of age, and also to a lesser degree in the longer periods.

There is a marked increase in the weight of the eyeballs in the test rats during both the short and the very long fasts.

The thyroid gland apparently remains practically unchanged in weight in the test rats at three, six, and ten weeks, but usually suffers a loss later.

The thymus shows a progressive decrease to about 80 per cent. loss in weight in the test rats at ten weeks, with still greater loss (up to 94 per cent) at later periods.

There is little change in the weight of the heart in the test rats at three weeks. At six and ten weeks there is an apparent increase of 13 and 27 per cent respectively. During the longer fasting periods the heart apparently loses weight.

At three, six and ten weeks the lungs show a loss of about 29 per cent in the test rats. At the later periods there is an apparent increase which is of questionable significance.

The liver shows an apparent increase of 16, 70 and 64 per cent in weight in the test rats at three, six and ten weeks, respectively. During the very long fasts it is variable, with a tendency to decrease considerably in the majority of cases.

There is an apparent loss of 49 per cent in the weight of the spleen in the test rats at three weeks, while at six and ten weeks there is an apparent increase of about 27 per cent. During the later periods the spleen is variable, showing a tendency to decrease in most instances.

The alimentary canal, including contents, shows a loss in the test rats at three weeks of age, whereas at six and ten weeks there is an apparent increase. The changes are variable in the longer periods.

There is a marked increase in the weight of the empty alimentary canal in the test rats up to ten weeks. A reduction in weight, however, usually occurs during the very prolonged fasts.

The suprarenals in the test rats show a progressive increase in weight amounting to about 114 per cent at ten weeks, which usually is maintained, to a lesser degree, during the longer fasts, in the majority of cases. At ten weeks of age and also at subsequent periods the suprarenals are heavier in the test females than in the test males of corresponding age and body weight, thus indicating the appearance of sexual differentiation in weight during underfeeding.

There is an increase in the weight of the kidneys in the test rats at three, six, and ten weeks of age, but its variation from the normal appears doubtful at later periods.

The testes show a marked increase in weight in the test rats at three, six, and ten weeks, but later are variable, in some cases showing no change, and in other instances losing considerably in weight.

There is an increase in the weight of the epididymides of 95 and 14 per cent in the test rats at three and six weeks respectively. At ten weeks and later the epididymides apparently lose weight in the underfed rats.

The ovaries show a marked increase in the test rats at three, six and ten weeks of age, but are variable later, in most instances showing a tendency to increase.

There is an increase in the weight of the hypophysis in the test rats at three, six and ten weeks. Later the hypophysis is variable, showing a loss in the males and an increase in the females in most instances. In the very old test females the weight of the hypophysis is higher than in the corresponding males, indicating that sexual differentiation in the weight of the gland had occurred.

There is apparently no definite change in the weight of the pineal body during either short or very long periods of fasting.

In general, it therefore appears that in rats underfed from birth up to three, six and ten weeks, there is a marked increase in the weight of the spinal cord, eyeballs, liver, and stomach and intestines (empty). A less marked tendency to increase occurs in the brain (especially in the earliest period), heart (progressive increase), spleen (at six and ten weeks), intestinal contents (at six and ten weeks), suprarenals (progressive increase), kidneys,

testes, epididymi (at three and six weeks, loss later), ovaries and hypophysis.

There is no marked change in the weights of the thyroid and pineal glands. A marked loss in weight occurs in the lungs and especially in the thymus. In the earliest period only (up to three weeks) there is a loss in the weight of the spleen and intestinal contents.

In the rats underfed for very long periods (starting at three weeks of age), the well-marked increase is maintained in the weight of the spinal cord, eyeballs, and usually of the suprarenals. The brain also shows a slight increase. In the case of the lungs, however, the apparent increase is of doubtful significance. There is apparently no marked change (or inconstant variability) in the weights of the intestinal contents, kidneys, ovaries, testes (?), and pineal body. The thyroid, thymus, heart, liver (variable), spleen (variable), alimentary canal (empty), epididymi, and hypophysis (in males) usually suffer a loss in weight during the long underfeeding periods.

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EXPERIMENTS ON THE PHYSIOLOGY OF DIGESTION IN THE BLATTIDAE

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TWENTY-ONE FIGURES

INTRODUCTION

The following experiments were undertaken to explain certain phases of digestion in cockroaches, and especially the processes concerned in digestion of fat, as well as other related problems. The subject is not a new one; many investigators have studied various phases of the problem, but they have in several cases arrived at very different conclusions. One of these workers was Prof. Alexander Petrunkevitch ('00), who suggested the problem to me.¹

The real object of my investigation has been to attack the problem from a new point of view, besides repeating to a greater or less degree the experiments of previous workers. I have endeavored to amplify considerably the scope of previous work through the use of newer stains and the physiological and experimental methods.

The main problems which I hoped to investigate and elucidate were concerned with the rôle of the crop in digesting and ab-

¹ Prof. Petrunkevitch's paper appeared in 1900, and since then several investigators have come to results distinctly at variance with his. This disagreement is explained in part by the fact that Professor Petrunkevitch gave almost no data concerning the details of his experiments. Most of the data were lost in consequence of political disturbances, and without the data the work could not be defended. Moreover, lack of these data prevented repetition and verification of the experiments. After many trials I arrived at methods of experimentation which tended to verify Petrunkevitch's results, and to explain the disagreement of other authors. From this beginning I proceeded to further and more varied experiments, always using groups of animals in each experiment. More than six hundred cockroaches were used in all.

sorbing food, the various functions of the gizzard, the details and significance of digestive processes in the stomach and coeca, and the explanation of the presence of certain substances and leucocytes in the tracheal tubes.

MATERIAL AND METHODS

The material first used consisted in the species of cockroach previously used by most earlier investigators in the field of fat digestion in insects, namely, *Blatta orientalis* Linnaeus (*Periplaneta orientalis*), the common black cockroach, and *Blatella* (*Philodromia*) *germanica*, the croton bug. The latter species was found rather small for convenient dissection and observation. Later on I used *Periplaneta americana*, the American cockroach, almost exclusively. In this species, which is very hardy and much larger, it seemed hopeful that some processes which had been obscure or disputed in the smaller species might be seen more plainly, and indeed this proved to be the case. One especially fortunate fact was discovered, namely, that the body cavity in the abdominal region could be entered in the living animal by turning back a flap of the body wall. After the flap was replaced, the animal experienced no serious inconvenience. It was found possible thus to make ligations of the alimentary canal just after food was taken, thus isolating certain digestive organs from connection with others. This eliminated the uncertainty of previous work as to the flowing of digestive juices from one part to another. The digestion which occurred in a part tightly ligated from other parts was considered to be surely produced by digestive fluids and enzymes secreted in that part.

Another fortunate feature about *Periplaneta americana* is its tolerance of sunlight. Unlike *Blatta orientalis*, which is very sensitive to light and avoids it whenever possible, my animals walked around contentedly even in bright light and took food normally.

The tissues were usually fixed in Flemming's strong solution, which contains osmic acid and stains fats black by an oxidative process. This fixing fluid is especially effective in work with

olive oil, for the pure oil consists mostly of the glyceride of oleic acid, and oleic acid is the only one of the four common fatty acids of ordinary fats which is blackened well with osmic acid, this being due to its unsaturated molecular structure. In work with tissues containing blackened fat it seemed inadvisable to stain with any haematoxylin stains, for these stain so darkly and tend to obscure appearances of the darkly stained fat. As a general stain acid fuchsin was used successfully; it stains nuclear and cytoplasmic structures sufficiently distinctly. For obtaining the best histological details Perenyi's fluid was much used, and also a mixture of 5 per cent formol and 50 per cent alcohol. The best combination of stains for accurate definition of structures was acid fuchsin, followed by Ehrlich's haematoxylin. In working with the very compact and hard tissues of the gizzard, Flemming's killing fluid was very satisfactory when it penetrated well, which was not always the case. Perenyi's fluid was also satisfactory. In order to bring out the muscle structures of the gizzard, careful staining with iron haematoxylin and picro-fuchsin is necessary. When the animals had eaten fat stained with Sudan III or Nile blue sulphate, sections were made in a freezing microtome.

Most of the tissues contain so much water that extra care is necessary in running them through a series of reagents. In running ordinary tissues from absolute alcohol through xylol to melted paraffin, it is very desirable to interpose between the alcohol and xylol two appropriate mixtures of these reagents. In running from xylol to melted paraffin, it is necessary to interpose a cold and a warm mixture of the two. Unless this is done, the nuclei and cytoplasm shrivel and the fat globules become distorted and liable to fade rapidly.

The fat used as food in the experiments was pure olive oil. The animals refused to take pieces of solid fat, but would take bread soaked in olive oil. But the ideal food seemed to be a paste of pulverized sugar and olive oil. This was taken greedily, even when stained brightly with Sudan III, Nile blue sulphate, or litmus powder. The organs chiefly concerned in recognizing the food seemed to be the maxillary and labial palpi, not the eyes

or antennae. The feeding was done in covered glass jars, in one part of which some of the paste was placed. The animals, when put into the jars, walked or ran about until the mouth parts and palpi happened to touch the food; then stopped, moved the palpi over the food and began to eat by opening and closing the mandibles. After feeding was completed the animals were removed to a clean dish. When colored foods had been eaten, it was always possible to decide in a general way how much a given animal had eaten, for the color of the food in the crop showed through the transparent crop wall and the semitransparent region of the body wall between the third pair of legs. When an animal had eaten a moderate or very generous amount of the paste, it stopped to clean itself carefully, going over the whole of the anterior legs with the mouth parts, and drawing the antennae through the mouth parts by aid of the anterior legs, meanwhile apparently licking off any oil or other extraneous matter on them. The fat-feeding methods of Schlüter were also employed; that is, the smearing of the head and anterior body with olive oil with a brush and then relying on the cleaning habits of the animals to get the food into the mouth and crop. This method was considered inadequate and not practiced much, because it was so unnatural, and because experience showed that fat smeared on the thorax entered the thoracic spiracles readily, and thus gave false pictures of fat distribution in subsequent preparations.

ANATOMY AND HISTOLOGY OF THE ALIMENTARY CANAL

In view of the fact that there is a discrepancy of opinion as to the structure of the alimentary canal, it seemed desirable to investigate it once more without going into unnecessary detail. This was undertaken as a basis for physiological work. The comparative lengths of the various parts in a specimen where the total length of the alimentary canal was 6.7 were as follows: oesophagus 0.25 cm., crop 2.5 cm., gizzard 0.25 cm., stomach 1.3 cm., small intestine 0.25 cm., large intestine 1.6 cm., rectum 0.5 cm. It will be noted that the crop possesses by far the largest

surface of any single organ, being 2.5 cm. in length and on the average 0.6 cm. in diameter when distended with food, thus having a surface of about 3 sq. cm. as against a corresponding surface of 0.7 sq. cm. in the stomach, which is 1.3 cm. in length and 0.25 cm. in diameter.

The stomodeum, or that part of the anterior alimentary canal which is turned in during the embryonic period, is of considerable size in the American cockroach, and may be divided into six parts: mouth cavity, pharynx, oesophagus, crop, anterior gizzard, and posterior gizzard. The mouth cavity and pharynx were not studied, for it is most improbable that any important digestive changes occur there. The salivary glands are paired, one lying on each side of the crop along about half its length. The ducts run forward into the mouth and are strengthened by a tough spiral structure similar to that of the tracheal tubes. The reservoir of the salivary secretion may be very large, it may be almost as long as the crop when it is dilated with fluid.

Oesophagus. The oesophagus begins where the dilation of the pharynx ends and varies much in size according as it is dilated with food or not. When empty its cavity is nearly obliterated by closely appressed folds. These folds are continuous with those of the crop to such an extent that it is impossible to say where one ends and the other begins. The oesophagus wall consists of a single layer of nearly cubical epithelial cells (fig. 1), between which are seen muscle processes and tracheal end cells similar to those described by Petrunkevitch. Within the layer of cells and secreted by it is the chitinous intima, of about the same thickness as the cells. This is raised into projections, on the summits of which long bristle-like processes are situated. Around these structures is a layer of circular muscles.

Crop. The crop is by far the largest part of the alimentary canal and no doubt has an important function, though Schlüter considers it as a mere storage organ and conducting tube to the stomach. The wall consists of three layers, muscular, epithelial, and chitinous. The muscular layer consists of two layers of striated fibers at right angles to each other. One layer consists of circular muscles and serves to contract the crop walls

tightly over any food within. The other layer consists of longitudinal muscles. The muscles form a loose network, and through the meshes the general body cavity connects with spaces under the epithelial cells. These spaces are under folds in the epithelial layer in the usual state, but are nearly or quite obliterated when the crop is distended with food. In these folds are found muscle fibers and processes which run from the muscular layer to the epithelial and chitinous layers, and may be considered as radial muscles, as shown in figure 2. Wandering blood cells and tracheal branches are also found in these spaces (fig. 2). The latter extend on one side to larger tracheal branches and on the inner side to tracheal end cells.

The epithelial cells have the shape of hexagonal prisms twice to four times as high as broad. The nuclei are situated near the bases of the cells; that is, toward the body cavity, as shown in figures 2, 5, 17. The cytoplasm of the nuclear region usually stains more deeply than the cytoplasm of the other end of the cell, and is apparently more specialized. Between the cells and the lumen of the crop is the rather thin chitinous intima. Petrunkevitch has demonstrated that this intima is porous, and I have verified his work, using his methods. Some earlier workers used this porosity to account for the passage of fat globules into the cells from the lumen, but it is now known that fats cannot be absorbed as such, but must be split to fatty acid and glycerol, both of which, being soluble, may be absorbed into the cells.

The muscular layer may be separated to a greater or less extent from the epithelial layer by teasing with needles under a binocular microscope. This applies for the oriental cockroach, and not for the American cockroach. Flat preparations of the epithelium may be made by holding a piece of the crop's wall tightly down and flattening out folds under a coverglass, then running in Flemming's fluid, or absolute alcohol, as recommended by Petrunkevitch. A much better method of getting flat preparations consists in feeding an animal until its crop is distended and therefore all the folds of the surface are smoothed; the crop is then removed without puncturing and preserved in

Perenyi's fluid. In the American cockroach there is a definite region of the anterior crop where there are very few muscles, this region is especially favorable for study. In these preparations the epithelial cells appear mostly as hexagons.

Petrunkévitch has described certain unusual conditions in some of these cells, and has figured them as seen when flat and in cross sections. In some cases the cell walls of a group of cells degenerate and the cells fuse. The cytoplasm appears vacuolated and abnormal and the nuclei begin to degenerate. Finally the whole mass of protoplasm seems to be discharged and the gap filled by the growth and cell divisions of adjacent cells. He also describes cells which contain two or more nuclei, but do not appear abnormal otherwise; these represent small syncytia resulting from nuclear divisions with suppressed cytoplasmic divisions.

Among my preparations some slides show stages which may be likened to these. The cells show great inequality of size, the very small ones appear to be latent, but ready to replace by growth any gaps in the epithelium. Binucleate and trinucleate cells often appear, but I cannot explain their significance, perhaps they represent small syncytia. Some of them are shown in figure 18. Another unusual condition is shown in figures 17 and 18. In such cases the cytoplasm appears abnormal and numerous nuclei are present, some of them appearing degenerate or as mere vesicles. No traces of cell walls are present in these regions, and the cytoplasm seems dead. I have been unable to find a definite explanation of such pictures; perhaps they represent a sort of lesion which results from injury to the cells through mechanical damage by sharp pieces of food or through the action of poisonous matter in the food. The process cannot, I think, be compared with the normal casting of cells of the stomach epithelium after they have become worn out by continual functioning.

Petrunkévitch has also figured ring cells; that is, cells which contain a very large vacuole surrounded by a thin layer of cytoplasm containing the nucleus. He believed that these cells represented a stage in the fusion and casting of cells, especially

as two or three cells might combine to one ring cell. In the species I have studied this appears not to be the case. I have never found any such appearances except in preparations which were fixed in absolute alcohol, and even then the appearances were not distinct, and probably represented artifacts due to too rapid dehydration.

Gizzard. The gizzard consists of two distinct parts, an anterior and a posterior. Its shape is in general that of a cone whose blunt end is anterior and adjoins the crop and whose pointed end extends into the stomach. Its altitude about equals the diameter of the base. The hinder, protruding part is of about the same length as the anterior part. The two parts are entirely different histologically. In the anterior part the chitin forms six heavy teeth; the chitin has no distinguishable structure for the most part, but may be shown by staining with erythrosin to consist of three homogeneous layers, an outer and an inner layer which take no stain and a median layer between them which stains bright red.

The six thick teeth fill most of the cavity of the anterior part. They are large projections, triangular in cross section and roughly rectangular in longitudinal section, which nearly fill the space within, leaving only a narrow lumen in the middle and small spaces between their sides (fig. 8). The outlines of the teeth are not quite triangular, but often have swellings or concavities on the sides and somewhat flattened points. Usually a swelling of one tooth lies opposite a cavity on the adjacent tooth, and flattened and pointed ends do not sharply oppose each other. Ramme has found that in cockroaches the six teeth fit tightly and perfectly and are so well held together by the thick circular muscles that a tight fastening may be made.

Under the chitinous intima lies a single layer of long cylindrical epithelial cells, and below them a mass of connective tissue, through which run many tracheal tubes. Just under the epithelial cells these tubes end in tracheal end cells, whose processes run up between the cells above. Outside of the connective-tissue layer is a broad layer of circular striated muscles.

Between the main teeth are secondary and tertiary ones. These, unlike the primary ones, are covered with short spines. Between each pair of large teeth are three secondary ones, evenly spaced (fig. 9). Between each primary tooth and its adjacent secondary tooth are three or five tertiary teeth, and between adjacent secondary teeth are two or three tertiary ones. So the gizzard has six primary teeth, eighteen secondary ones, and sixty or ninety-six tertiary ones.

The teeth extend through about half the length of the anterior gizzard. The region between their posterior ends and the passage from the gizzard into the stomach is provided with smaller rounded projections which are set with long yellow spines or needles. Just behind the primary teeth are considerable folds, which Miall and Denny have called cushions for the teeth. These are shown in figures 8 and 9. The spines here are large and uniform, and are situated in sockets on the summits of dome-shaped chitinous projections. The structure and relations here will be discussed later. Posterior to the cushions lies a region of many smaller lobes and folds, the walls of which merge into the duct leading to the stomach. These lobes are thickly set with simply-structured bristles. Adjacent and opposite lobes are usually closely opposed to each other, so that little or no passage is left (figs. 8 and 15). They may thus effectually block the passage of food from the crop through the gizzard to the stomach.

The epithelium of the anterior part of the gizzard merges into that of the posterior. This posterior part projects far into the stomach and consists of one epithelial layer bounding the lumen and another which turns back from the apex of the projection parallel with the first and outside of it. This epithelium merges with the extreme anterior end of the stomach wall, in the region where the coeca originate. The lumen of the projecting part of the gizzard is small and is almost obliterated, being reduced to a star shape by the projecting rounded folds of epithelium, which are surrounded by muscles. Occasionally I have found small black bodies in the cells here in preparations fixed in Flemming. The epithelium of the outer side bears small rounded chitinous bodies to which short spines are attached.

Stomach and coeca. The stomach extends from the gizzard to the point of entrance of the Malphigian tubules. In its normal position it is curved, and bent so that its posterior end is almost directly below its anterior end. It is crowded in the abdominal cavity, and is small in proportion to the size of the animal and in proportion to the crop's size. From the anterior end arise eight coeca, each of which is of about a third the diameter of the stomach and of about half the stomach's length. The variation in size is very great, especially depending on whether the coeca are filled with food or not. Sometimes the coeca are as long as the stomach. The outer wall of the stomach is composed of a loose meshwork of muscular fibers, among which tracheae ramify. The same can be said for the outer wall of the coeca. The assertion of Jordan that the fibers of the muscle meshes only pass under unspecialized cells seems to apply in the American cockroach. In this way much of the surface is left free of striated muscle, which would, if present, hinder the passage of food materials from the epithelial cells into the body cavity.

The epithelial cells are in general long and narrow as seen in either transverse or longitudinal section, and are similar throughout the whole length of the stomach. As in most insects, the inner edge is set closely with fine filaments which serve to protect the epithelial surface (fig. 21). At very frequent intervals groups of immature cells are seen between the true epithelial cells and the muscular layer. These cells are small and closely packed. In many places all intermediate stages between them and adult cells are seen, as shown in figure 21, so it seems certain that there is a continual replacement of the mature cells, which often die and are cast, as I shall later describe. The epithelium usually shows but few adult cells in a group, and these groups more or less widely separated by the intermediate cells which take their places later. The surface of the epithelium may be flat or undulating, but is often raised into small villi with U-shaped spaces between them, the so-called crypts of Frenzel. The ends of the villi may be smoothly rounded or bulged out considerably. The arrangement in villi allows a much greater surface for secretion and absorption. I agree with Biedermann that the crypts

are not glandular in function, but are adapted for efficient regeneration of cells. The membrane lining the wall of the epithelium is evidently cast at intervals, for it may be seen at various stages of disintegration. Such stages correspond to those described by Schroder. The spheres of secretion which appear on the surfaces of the cells will be discussed in a later section on the functions of the stomach.

The structure of the epithelium of the coeca is the same as that of the stomach in every detail. This shows plainly in sections which show the branching of the coeca from the stomach. In these cases it cannot be determined just where the coeca originate. I have seen practically no evidence of more secretion globules on the coeca epithelium, an observation which is surprising in view of the fact that several authors have described the coeca as preeminently secretory organs, capable of producing most of the secretion found in the stomach. Later I shall show that not only are the histological structures of the stomach and coeca alike, but also the digestive functions.

At the hinder end of the stomach the epithelium makes a circular fold which partially constricts the passage into the small intestine. This may be considered as a valve. Schroder suggests that the purpose of it is to help roll up boluses of food matter and pass these on to the small intestine, instead of a constant stream. Backflow is somewhat prevented, too.

Small intestine. The small intestine is very short and has a small lumen. Just behind the fold or valve at the hinder end of the stomach the high epithelium of this region gradually becomes lower and merges into the thin epithelium of the small intestine. At this point the Malpighian tubules enter in six great groups. The epithelium in this section consists of nearly cubical cells whose cell boundaries are hard to make out. The layer of cells is very much folded. The chitinous intima bears small swellings surmounted by very short spines. The cell structure resembles that of the oesophagus. Surrounding the epithelium is a thick layer of apparently confused muscular fibers. The function of the small intestine is not absorptive, and it has never been so considered except by Frenzel and Deegener. The explanation of

their findings may lie in their considering cells immediately behind the stomach valve as intestinal cells, while they really belong to the stomach.

Colon. The large intestine or colon is long and coiled in its position in the body. Its epithelium consists of long, narrow cells which are thrown into rather regular folds. Under the folds tracheae and tracheal end cells may be seen. In the American cockroach the histological details may be seen well after using Flemming's strong killing fluid. On the contrary, in the oriental cockroach, the structure is spoiled by Flemming's fluid, as Petrunkevitch and Schlüter agree. In any case Peryny's fluid is satisfactory. The colon is divided from the small intestine by a fold or valve of the epithelium. The intima is raised into significant mounds over each of the cells, but no hairs are evident, as described for *Blatta orientalis*. The function of the colon is not absorptive, at least I have not found it so, nor have many other authors except Frenzel, and his observations have since been disproved by later investigators. Simroth and Mingazzini have stated that certain sacs of the colon were absorptive in *Lamellicorn* larvae, but their descriptions indicate that there was no real absorption, but mere passage through pores. Berlese has described a similar case.

Rectum. The rectum is the last part of the digestive canal and terminates at the anus. It is surrounded by a thin muscular layer. The so-called rectal glands are six lobes of very long cells which bulge far out into the lumen. Their cells do not quite reach the muscular layer, but leave small interspaces, in which tracheae ramify. The cells of the glands have a distinct intima, but no chitinous filaments. This intima is continuous between the glands and dips down almost to the muscular layer, there being almost no cells between:

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Oesophagus. The function of the oesophagus is merely to conduct food into the crop or to retain it for a time if the crop is quite full. The thick intima evidently precludes all secretion and absorption here. No globules of absorbed fat were ever seen in the cells.

Salivary glands. Various investigators have found that the reaction of the saliva in Orthoptera is never acid, but may be neutral, as Plateau found in *Blatta orientalis*, or alkaline, as in *Blattella germanica*. Jordan states that the saliva has about the same digestive power on carbohydrates that human saliva has.

Crop. If we consider the function of the crop, there are two principal possibilities, 1) secretion and 2) absorption. A third possibility is storage of food, and this is no doubt an important factor, enabling the cockroach to store here much of a very large meal and utilize it during a foodless period of several days or weeks, especially during bad weather in its native home in the tropics. The large size of the crop is evidently an important factor in making them as hardy and prolific as they are. This is in accord with Jordan's theory that the manner of life brings about more variation in the foregut of insects than in the mid and hind guts.

I have almost never found the crop entirely empty, though after three weeks of starvation in a clean glass jar it may contain nothing but a small amount of fluid. Under such conditions, they ingest various non-nutritious substances, such as wood shavings and tarsi and pieces of antennae of their mates. The content usually appears as a sticky gray mass. After they had eaten all they could of the red mass I fed them, their crops were very much distended, completely filling the thoracic cavity and more than half of the abdominal cavity, extending as far back as the fifth abdominal segment. The pressure caused the epithelium to bulge out between the muscle strands. Frequently this pressure was so great that a part of the food was regurgitated. Such a meal may last the cockroach two months or somewhat less, as found by dissection at various intervals. This surely shows that the crop is important as a storage organ.

The epithelial cells are all alike histologically and show no special characters of secretion or absorption. Experiments to be described herein showed that secretion occurred in these cells, but methods of direct observation proved insufficient to determine the reaction of this secretion. The reaction of the content of the crop depends largely on the kind of food in the lumen, partly on the

secretion of the crop cells, somewhat on the secretion which flows in from the stomach, and partly on the secretion which the salivary glands have poured in. It is certain that in the crop the neutral or alkaline secretion of the salivary glands acts on carbohydrates and renders them soluble, as Jordan and others have established.

To obtain information as to the reaction of the secretion of the epithelium of the crop alone, I removed the crops from ten animals and placed them in normal salt solution, then slit them longitudinally, and carefully washed out the contents. The crops were then ground fine in a mortar with 10 cc. of clean salt solution, and the mixture left two hours to make an extract. The mixture was then filtered and the filtrate tested. This filtrate proved to be neutral to phenol-phthaleine and alkaline to litmus. This indicated definitely that the normal secretion of the crop is slightly alkaline.

Though the crop secretion is alkaline, I did not always find in my feeding experiments that the contents of the crop were alkaline after fatty food had been eaten for certain periods. Nile blue sulphate is a good indicator in work with fats, for it stains fats blue and fatty acids red. I fed an animal a large amount of a paste of olive oil and sugar, stained bright blue with the indicator stain. Three days later, on dissection, the whole content of the anterior crop proved to be deep red, while the epithelium was blue. The presence of red fatty acid in the lumen indicated that some agency, probably an enzyme, had acted on the fat to produce fatty acid. This process was more definitely explained by a series of feeding experiments in which powdered litmus was mixed with the food eaten by the animals. If, when the crop was subsequently opened, the contents were observed to be red, they were considered to be acid in reaction; if blue, alkaline.

Miall and Denny, Ramme, Jordan and Biedermann have maintained that the acid secretion found in the crop after feeding was derived entirely from the stomach and coeca, which had flowed from them through the gizzard into the crop. Their idea is that a large part of the digestion occurs in the crop, but that

the enzymes came all originally from the stomach, coeca, and salivary glands.

My investigations indicate that the findings of the above investigators are partially correct, but not entirely so. The crucial experiment was the following. An almost starved roach was fed a large amount of a mixture of olive oil, powdered sugar, and blue litmus powder. Immediately afterward the abdominal cavity was opened and the crop tightly ligated just anterior to the gizzard. The operation was done carefully and without much inconvenience to the animal. The parts were replaced in as nearly natural conditions as possible, and the animal was left for twenty-four hours, then dissected in salt solution. The colors of the contents of the various parts were observed to be as follows: crop anterior to ligation, red and some blue merging into red; crop at ligation, blue; crop posterior to ligation, deep red and a little blue merging into red. The redness of the crop posterior to the ligation indicated, no doubt, that acid secretion had migrated thence from the stomach and through the gizzard. The distinct blueness at the ligation point indicated that the ligation had been successful, for if it had not been, the acid secretion of the stomach would have flowed in through the gap and the color would have been red instead of blue. The explanation of the redness anterior to the ligation is an interesting problem. Redness here means acidity, and the real question has to do with the origin of the acidity. Four explanations seems possible: 1) that some stomach secretion got into this part of the crop; 2) that the saliva from the salivary glands caused the acidity; 3) that the reaction of the food itself was acid, and 4) that the acidity was caused by the action of some secretion or enzyme of the crop itself. The first possibility need not be considered, for the tight and successful ligation, as mentioned above, prevented any flow from the stomach to this region. The second possibility is not valid, for all investigators hold that the saliva is alkaline or neutral. The third possibility is ruled out by the fact that some of the original paste was left in the feeding jar all through the experiment, and was as blue at the end as at the beginning. So the fourth possibility seems to be the true one, and the acidity

must be considered as derived from secretion or enzyme action of the crop. If it is derived from a normal and usual acid-secretion flow, this secretion should be present after feeding of various foods. To test this a roach was fed a large amount of a paste of mineral oil, powdered sugar, and blue litmus powder, then the body cavity entered and a ligation made as before, and the animal left twenty-four hours before dissection. The experiment was thus exactly the same as the preceding except for the substitution of the indigestible mineral oil for the digestible olive oil. In this second specimen it was found on dissection that the crop posterior to the ligation contained deep red material just as it did in the first specimen; but the crop anterior to the ligation was completely blue. We must now explain why acidity occurs in the crop after olive-oil feeding, but not after mineral-oil feeding. All other possibilities having been ruled out, it seems that the acidity must have come from some product of the olive oil. This oil is neutral in reaction, but one of its digestive products is acid, namely, the fatty acid, in this case mainly oleic acid. This acid seems to me to be the only possible cause of the acidity observed, and if it is present it indicates that the fat (olive oil) has been split to its components in the crop and under the influence of the crop alone, other possible influences having been eliminated. The fat-splitting enzyme of the alimentary canal of animals is lipase. There seems good evidence that this lipase is secreted by the crop. Its purpose is to prepare the insoluble fats for use by changing or splitting them to soluble products, capable of diffusion.

The real test of a digestive process consists in digestions carried on outside of the body. This method also allows quantitative calculations and affords an actual and true comparison of the digestive power of various organs. It also gives an opportunity to decide whether Petrunkevitch was right in asserting that the crop is the most important organ in fat digestion or whether it is a mere subsidiary to the stomach, as so many authors have asserted. Trials show that artificial digestions may readily be carried on. The test of the amount of digestion consists in titration against alkali. When fat is digested fatty acid is produced,

and the amount of acidity present at any stage depends on the fatty acid present, and is a measure of the extent to which fat has been split, or, in other words, how far its digestion has proceeded. Thirty animals were used in the crucial experiment. They had been starved for varying periods, so that little food was present in the crops or stomachs. All receptacles used were cleaned by boiling at the beginning of the experiment and physiological precautions were observed. The animals were all carefully dissected, the crops and stomachs removed, the crops put into one dish of normal salt solution, and the stomachs with their coeca into another. After the salivary glands were removed, the crops were slit longitudinally, and the contents carefully washed out with a soft brush. The thirty crops were rinsed and ground to a pulp in a mortar, 30 cc. of normal saline being added. This was left for two hours while the enzyme was extracted, then the solid matter was removed by filtration. The filtrate (about 25 cc.) was made up to 30 cc. with saline and divided to three portions of 10 cc. each in flasks. One portion was titrated at once against 1/20 normal NaOH, using phenolphthaleine as an indicator, and found to be neutral. The flask containing another portion of 10 cc. was plugged and left three days at room temperature in darkness, then titrated. This showed an acidity corresponding to 0.1 cc. of the alkali. This slight increase in acidity over the original amount was due to bacterial or other causes. To the third flask of 10 cc. of the extract was added 10 cc. of pure olive oil (neutral by titration tests), the mixture was left three days in darkness at room temperature and shaken at intervals. It then showed by titration an acidity corresponding to 10.8 cc. of 1/20 normal alkali. Comparing the flasks with and without olive oil, there has been an increase in acidity corresponding to 10.7 cc. of alkali. The two flasks contained the same amount of the same extract, and were treated similarly, but for the addition of the oil to one. Thus the acidity must have been derived from the olive oil, and must represent the presence of fatty acid as a product of the oil. The presence of fatty acid indicated that splitting of the fat had occurred during the experiment, or, in other words, that digestion of the fat had

occurred outside of the body by the enzymes derived from the cells of the crop's wall.

The thirty stomachs with their coeca were in general treated like the thirty crops. Their contents were pressed out through the open ends, and the coeca were punctured when it was impossible to remove their contents otherwise. The grinding, extraction of the enzyme, filtration, and division to three portions of 10 cc. was performed as described for the crops. Immediate titration of one portion showed an acidity corresponding to 0.3 cc. of 1/20 normal alkali. The other two portions were left three days as before, oil being added to one. The portion without oil titrated against 1.0 cc. of alkali, showing an increase of 0.7 cc. due to bacterial or other action. The portion to which 10 cc. of oil had been added titrated against 7.2 cc. In this case, then, there was an increase in acidity of 6.2 cc. over the control. This increase must have been due to digestion and production of fatty acid, as just discussed for the crop. This is just what all investigators would anticipate, I think, for all have considered the stomach as an important organ for the preparation of food for the uses of the body.

The above results show without question that fat is digested in the crop and stomach. They also afford us comparative values for the importance of the digestion in the two organs. The figures indicate that the crop is more important than the stomach in the proportion 10.7 to 6.2. I shall later show that more than half of the effect of the stomach with its coeca is caused by the coeca, so the results really indicate that the crop is between three and four times as potent in fat digestion as the stomach per se. All question of individual variation is obviated by the fact that thirty animals were used together in the experiment. The crop is therefore the chief organ of digestion of fats, just as Petrunkevitch stated in 1900.

In other experiments some extracts of enzyme were boiled. These were compared as to digestive power with unboiled ones. Such boiled preparations showed much less acidity in later stages of fat digestion than unboiled ones. The fact that the active agent of the digestion is destroyed by boiling is additional evidence of its enzymatic nature.

I attempted to use the soluble fat ethyl butyrate in a series of experiments similar to those described. In every case the acidity at the end of the experiment was about the same as that at the beginning, so there was evidently something toxic about the fat which resisted the enzyme, as other workers have found in vertebrates.

Absorption in the crop is not a generally accepted view at the present day. No author of the last decade has spoken decidedly in its favor; nevertheless, I believe it regularly takes place and is of very large importance. Plateau was the first careful investigator in this field; he concluded that in the form he studied a large part of the absorption of food occurs in the crop. Jousset de Bellesme found similar results in his experiments with sugar, in which he seemed to prove that sugar entered the cells from the lumen by diffusion. Both of the latter investigators found evidence that starchy food does not soon pass into the stomach, but is retained by the crop to be digested and absorbed. In criticism, Cuénot and Biedermann have sought to disprove this by assuming that there is a continual passage into the stomach and subsequent rapid absorption; therefore but little is ever present in the stomach's lumen.

Cuénot observed that the cells of the crop have a chitinous intima, while those of the stomach do not, so he reasoned that absorption should only occur in the stomach. His conclusions were not entirely verified by experimental work, so they cannot be accepted unreservedly. He fed colored fluids to *Blatta*, and found them after several days present in the stomach only. I have never been able to repeat this experiment, and I believe we must conclude that some of the colored matter he fed was absorbed by way of the crop during the several days the experiment lasted, so none was present there when the animal was dissected.

Petrunkevitch in 1898 made a series of experiments in which he starved cockroaches twenty-four hours, fed them lumps of fat, and killed them after varying lengths of time. Large amounts of fat were found in the epithelial cells, and progressively less with longer and longer periods afterward. The periods of time used are

not given. In some cases the cells were found so full of fat that they appeared as solid black, excluding all other structures from view. The fat found in the cells had evidently got there from the lumen of the crop, seemingly through pores. It was really a clear case of absorption by diffusion. In late stages of digestion fat globules were found lying free in the pouches of the body cavity behind the epithelial folds.

On the basis of Petrunkevitch's work, Sinety attacked the problem. He fed with fat fifteen hours and found fat beginning to appear in the cells. This is said not to be of special significance, but to represent fat which is absorbed from the blood by the cells and stored in them as reserve food material. His belief is that these reserves explain Petrunkevitch's figures.

Schlüter also based his work on that of Petrunkevitch, but used a special feeding method, namely, smearing of fat on the body and reliance on the cleaning activities of the animals to get the food into the mouth. He found that fat appeared in the cells and also in the blood lacunae fifteen to twenty minutes after feeding began. He assumed that most of this must have gotten through the intima and cells unchanged, for the splitting of fat to soluble products and their absorption could hardly have occurred so soon. The appearance was only seen in those parts of the crop where fat actually lay against the cells. A complicating factor was some residual fat, which was present in the cells when the experiment began. He never found cells completely filled with fat, but did find places where fat had rushed in from the lumen through a hole in the intima and had filled cells. Thus he explains Petrunkevitch's appearances of completely filled cells. In other experiments he starved animals eight days and fed them fat one to three days. None was subsequently found in the cells, but it was found there after eight days of sugar feeding. This led to his assumption that fat is made from carbohydrate in the body and is sometimes stored as fat in the cells.

Jordan in his text-book on digestion in invertebrates admits that much carbohydrate may be absorbed in the crop, but is doubtful as to fat, for the work of Sinety and Schlüter indicates that fat in the cells may be a product of metabolism.

In my experiments I used animals which had been starved two weeks to be sure no residue of fat was in the cells. As a matter of fact, no intracellular fat could be found after six days' starvation. The animals were fed a paste of olive oil and powdered sugar, and were dissected and fixed in Flemming's fluid after varying lengths of time, prepared by the paraffin method, and stained with acid fuchsin. In some cases Flemming's fluid was injected into the body cavity, but as this method gave no better results it was discontinued. In one series of experiments in fat breeding the following conditions were found in the cells of the crop: eight hours after feeding, small amounts of fat in the cells; after sixteen hours, small amounts; after twenty-four hours, moderate amounts; after thirty-two hours, large amounts; after forty-eight hours, maximum amounts, with many large globules of fat in the cells and in some cases the cells entirely black with fat to exclusion of all cell parts; after seventy hours, large amounts, the globules large and often seemingly in process of dissolution (figs. 3 and 4). At later stages more or less fat is present in the lacunae behind the epithelium. Controls were tried with a paste of sugar and mineral oil, the latter oil being indigestible, but without getting pictures of fat globules in the cells.

The following experiment also has an interest in this connection. A cockroach was fed a large amount of a paste of olive oil, sugar, and blue litmus powder, then its body cavity was entered and its crop ligated posteriorly. After forty-eight hours the animal was killed and the alimentary canal preserved. The crop showed a red content anterior to the ligation and this part was preserved in Flemming. Histological examination of transverse sections revealed many globules of fat in most of the cells. This fat had been absorbed here, and prepared for absorption by the crop secretion, for the stomach secretion could not have gotten here past the ligation.

To test whether fatty acids really could be absorbed, oleic acid was fed in a paste with sugar. The animals were dissected at intervals and their crops preserved in Flemming's fluid. Black globules of osmicated fat were readily found in the cells

as follows: after eighteen hours, small amounts; after thirty hours, moderate to considerable amounts; after thirty-two hours, large amounts (fig. 6). So oleic acid is readily absorbed into the epithelial cells. The absorption is more rapid than in the case of fats, as might be expected, for fats must be split before absorption, while with oleic acid this is unnecessary.

In order to discover the relation between fats and fatty acids during the digestion of fat, a paste of sugar and olive oil was used as food; into the paste a generous amount of Nile blue sulphate was stirred. After three days the animal was dissected and the crop sectioned in a freezing microtome. The sections showed blue globules of fat in the epithelial cells. These were surely fat, for the stain colors fat blue, fatty acids red. Within the lumen of the crop and sticking to the intima a red mass was found, this was fatty acid derived from the fat eaten. In another case oil was stained bright red with Sudan III and fed. Subsequent frozen sections showed many red globules in the epithelial cells.

I have already stated that in some cases a large meal of fat may not all be utilized until about two months later. In these cases there is a continual absorption of fat in the crop throughout the whole period, as I have demonstrated by making a series of preparations at various intervals through the period of utilization of a crop's content of fat. Figure 5 shows a stage two months after a large meal was eaten. Absorption is almost completed, though some recently absorbed globules are seen in the region of the cells nearest the lumen. The middle region of the cells is occupied by large globules. The material of the globules is apparently being utilized, for the region between them and the body cavity is occupied by many small globules. These represent fatty material in process of passage (as soluble products) from the large storage globules to the blood in the body cavity. The large globules must represent storage products, ready to be utilized when needed. We have already seen that the lumen of the crop is important for storage of food, and now we see that the epithelium of the crop is important for storage, too, but in another sense.

A general survey of the facts I have described about absorption leads to the conclusion that the process of digestion of fat in the crop follows the usual course: fat is acted upon in the lumen of the crop by a lipase which in all probability comes from the cells as a secretion. This splits it to fatty acid and glycerol in the ordinary way. The soluble products are absorbed and re-synthesized into fat by the cytoplasm of the epithelial cells and used by the body for its nourishment. As a first step in utilization, the fat passes from the cells to the blood plasma in the spaces behind the epithelium, and from here is carried to all parts of the body by the blood.

There is absolutely no doubt that the presence of fat globules in the epithelium indicates that the cells have absorbed fatty material from the lumen. The globules are practically always present at appropriate intervals after fat is eaten, and appear uniformly throughout great regions of epithelium, giving the same pictures through whole sections and whole series of sections in dozens of preparations. The globules may be seen in flat preparations and in transverse sections equally well, and may be demonstrated by staining with osmic acid, Sudan III, and Nile blue. Schlüter would have us believe that any fat in the cells had got there from the lumen directly through slits in the intima. This might occur occasionally; but an examination of my figures and my several lines of proof as given above, together with the great uniformity of my preparations and their agreement with those of Petrunkevitch, makes it absolutely certain that the cells of the crop absorb fatty matter in large amounts, and exhibit more and more of it in form of globules as digestion progresses.

When a comparatively small amount of fat is eaten, it does not fill the lumen, but leaves part of the epithelium untouched. This untouched part shows no absorption stages, while the cells on which fat lies show fat within. Schlüter found the same condition. This fact is additional evidence of the absorptive power of the crop; if the fat reached the crop by way of stomach and body cavity (as several authors contend it always does) it would surely appear fairly equally in all parts of the crop wall, and not merely in those regions which contain fat within.

Schlüter has emphasized that sugar may be synthesized to fat in the insect body. Such a process can in no way effect the explanation I have given of fat globules in the cells, even though sugar was always present in the foods I fed. We have seen that a mixture of sugar and mineral oil used as food gave no subsequent fat in the cells; if sugar could be readily synthesized to fat in these cells, the paste with sugar and mineral oil should give the same pictures as the paste with olive oil. This is not the case. We may conclude that all intracellular fat is derived from the fat of the food.

I have obtained indirect evidence that sugar is absorbed in the crop. Animals which had eaten a large amount of a thick paste of sugar and oil were dissected after two days. The food matter found in the crop consisted of clear, liquid fat only. The sugar of the food had gone, probably through absorption by the cells of the crop. The experiment suggests that the absorption of sugar occurs rapidly and in large amounts.

Gizzard. The large teeth of the anterior gizzard would suggest a grinding function, especially since they are surrounded by a thick layer of circular muscles. Plateau was the first investigator of the significance of the gizzard; he came to the conclusion that it served as a filter with a narrow lumen, the various chitinous projections, bristles, spines, etc., serving to hinder the passage of food. He admitted no trituration, for he found full-sized fragments of plant food and uninjured starch grains in the stomach after passing through the gizzard. Jordan and Ramme both state that the teeth move, not for masticatory processes, but for the mixing of food with enzymes and for crushing food particles to only a slight degree. The mixing in of the enzymes quickens digestive processes by aiding penetration. Mingazzini reached his conclusions from general biological considerations, and states that the complicated gizzard of Orthoptera cannot be a mere filter. This view is expressed by Biedermann in Winterstein's Handbuch. It is partly based on the assumption that such rapid eaters as cockroaches must needs have some chewing device. However, I have already described the retention of food from passing the gizzard for several days after a rapidly eaten meal.

In my experiments I have never fed food consisting of large particles, so of course I have never observed any appearances of mastication. Food is almost never found between the points of the teeth. I have found considerable pieces of chitin in the lumen of the stomach; some small ones are shown in figure 19. These must represent relics of food which passed through the gizzard without being crushed much. I therefore agree with the majority of authors on the subject in not emphasizing the chewing action of the teeth. As the contour of the teeth may be such that they fit very compactly together, this part of the gizzard may act as a sphincter, though not as a perfectly tight one. Figure 8 shows the spaces which are usually present between the teeth. It is not here that the main sphincter action of the gizzard resides.

Food is found between the teeth and also in the regions of the secondary and tertiary teeth and other projections. The food is evidently guided from crop to gizzard by following in the narrow channels between the projections. The teeth must open somewhat to let the larger particles pass. The movement is peristalsis of the crop, which is often very powerful when observed in the living animal.

As mentioned in the section on the crop, the gizzard is of importance in conducting forward the secretion from the stomach. For I have found an acid secretion in the hind crop behind ligations, and this seems to have flowed thence from the stomach. How the passage forward through the gizzard occurs has not been determined, but it seems probable that it flows in the narrow channels between the projections. These channels are the ones used later by the food in its passage backward. I have endeavored to demonstrate the forward passage of stomach secretion by injecting colored fluids into the stomach of the living animal. Such operations have always proved rapidly fatal.

Ramme studied the flow of the secretion in various Orthoptera, but not in the American cockroach. In some he found special passages which led from the origin of the coeca directly into the rear gizzard. I have studied serial sections of this

region in the species I used, and have uniformly found no evidence of such passages. It may be that in some Orthoptera the coeca are special organs of secretion, so that a more direct passage would be advantageous. In the cockroach the coeca show the same secretory power as the stomach; a direct passage for their secretion would, therefore, have no significance.

The cushions of Miall and Denny deserve special attention. They are six in number and situated directly behind the primary teeth (figs. 7 and 10). Their surface is provided with needle-like spines, and these are especially significant on the surfaces directly toward the lumen. A definite band of striated muscle runs from this surface outward and upward to be firmly attached to the thick chitinous intima of the anterior surface of the teeth (fig. 7). Wilde has described the cushions as normally closed, their opposed surfaces fitting to form a tight joint. He considered that the tight closure of the cushions prevented the passage of food until it is well ground by the teeth. Basch thought that the cushions not only pushed against each other, but also pushed up closely under the teeth. Ramme has also described a close joint by the pressing together of the cushions by action of the surrounding ring muscles. Petrunkevitch has described the needles of the exposed surfaces as being attached inwardly to muscle fibrils, one fibril running to each needle. He believed that the contraction of the muscles caused movement of the needles by virtue of the attachment of the muscle fibrils to the bases of the needles. Ramme has ridiculed this view; he finds the muscles attached to the intima, not to the spines.

I have made many series of transverse and longitudinal sections of the gizzard, and never have I found the cushions closely appressed, and only in one case were they near each other. The cushions appear well separated from each other, as I have figured in transverse and longitudinal sections (figs. 7, 10, 15). As my preparations represented every stage of feeding and starvation, we may safely conclude that in the American cockroach the close opposition of the cushions occurs rarely or never and has no significance in the retention of food. The cavity of this

region is practically always open for the passage of food. The appearances are much clearer if studied in longitudinal sections; such sections were evidently not used by most investigators. For the study of the intimate structure of the cushions great care in preservation and the changing of reagents is necessary. Flemming's fluid often causes a loosening of the intima and the destruction of its structure. Any preserving fluid may do this, or insufficient dehydration, or too rapid passage into paraffin. Only after many trials was I able to demonstrate the fibrillar structures leading to the needles as Petrunkevitch has described them. I believe that the other investigators took insufficient precautions with the material, thus destroying the intimate structure. Flemming's fluid is satisfactory if the material is left in it two days; Perenyi's fluid may also be used. It is necessary to use iron haematoxylin as a stain, followed by picro-fuchsin. Even then many preparations do not show the desired structure, for the fibrils and their connections are to be seen only in a limited area, and it is only by chance that sections can be made in just the right plane. In well-preserved preparations it is clearly seen that the muscles of the muscle band divide to fibrils (fig. 11) which pass between the epithelial cells and become little tendons. The tendons traverse the modified intima to attach to the socketed bases of the needles.

The muscle bands are six in number, and run from the intima of the cushions outward and upward to their origin in the thick and comparatively immovable chitin of the anterior surface of the teeth. The muscles are long and well striated; their contraction can cause little movement at their origins, so it must result in movements at the insertions in the cushions. The insertions are in the bases of the needle, and the sockets seem to allow them to move in response to muscular pull. Thus the structure certainly indicates that the muscles move the needles. A cross-sectional view of the muscle tendons which traverse the intima may be seen in sections cut nearly parallel to the surface of the intima, and just below its surface. Figure 12 shows such a picture: the tendons are seen within the matrix of the intima in the region between the epithelial cells and the surface

of the intima. Such appearances disprove beyond question Ramme's contention that the muscles terminate in the inner surface of the intima. The fibers run not only to the intima, but through it (as tendons) to its outer surface and to the needle bases (fig. 11).

The needles are quite considerable in size, being 0.10 mm. to 0.15 mm. in length. Ramme has said that their extreme minuteness in comparison to the muscle structures precludes any mutual action, yet an examination of my figures reveals that the needles are much larger than the muscle fibrillae and tendons, and so connected with them that their movement by the tendons seems probable. The purpose of this movement is difficult to explain, but their position and arrangement suggest that probably they aid in moving food along through the gizzard toward the stomach, as Petrunkevitch suggested.

The region between the cushions and the narrow stomach passage is occupied by many folds of various sizes (figs. 7, 15, 16). These are all provided with spines, many of them being so inserted that they can lie flat on the surface. Several of the folds usually lie closely together, blocking entirely or partially the passage into the stomach. This arrangement enables them to hold back food, and I believe that in these folds the sphincter action of the gizzard is largely to be found. It is certain that the gizzard does act as a very efficient sphincter, holding back fluids for three days. We have seen that neither the teeth nor the cushions make a tight joint, so the only possible position for efficient retention of food is this rear part of the gizzard. Moreover, this is a logical position for a sphincter, for it is only necessary to protect the small opening into the stomach, the narrowest section of the whole alimentary canal; while in the more anterior gizzard, a much broader cavity would necessarily be closed by any structure acting as a sphincter. The arrangement of the folds is shown in cross section in figure 16, and in longitudinal section in figure 15. In both cases it is seen that the folds are surrounded by a considerable muscle sphincter, which brings about the appression of the folds by its contraction. The sphincter may be thicker than that which surrounds

the cushions. Its position seems to indicate that it is the effective agent in making the gizzard the strong sphincter organ which it is.

Jordan states that in some insects the posterior, projecting part of the gizzard may project completely through the stomach when it discharges food, so that the food enters the small intestine and not the stomach. This adaptation results in protecting the stomach cells, which have no chitinous wall like other parts of the alimentary canal, from actual contact with the food. In the American cockroach I find that these conditions do not hold. Two facts bear out this conclusion: 1) longitudinal sections of gizzard and stomach show that the gizzard projects only a short distance into the stomach; even if the epithelial folds were flattened out it would fall far short of extending through the stomach; 2) when food which can be recognized is fed, it can be later found and identified in the stomach cavity. Therefore, it is certain that the function of the projecting gizzard is not to protect the stomach.

Ramme's idea of the function of the projecting gizzard is quite the opposite of Jordan's. He worked on cockroaches and other insects and decided that the projecting part does not serve to protect the stomach epithelium, but to direct food into a region of the stomach well below the coeca, these being diverticula of the extreme anterior end of the stomach. He asserts that the coeca are secretory in function, and that the hinder gizzard by directing the food into a region well behind them prevents their being clogged with food and so hindered in their secretory processes. I shall show that the coeca are not special secreting organs. Moreover, the fat I fed did enter the coeca in large amount. So the projecting gizzard does not serve to protect the coeca in Ramme's sense. It seems likely that the arrangement of parts prevents the hard and sharp pieces of food from passing into the comparatively delicate coeca.

Stomach and coeca. Various investigators have discussed the question of secretion in the insect stomach, and especially in that of the cockroach. The first phase of the question con-

cerns the reaction of the secretion which is formed. Perhaps the first investigators in this field were Zuerst and Basch in 1858, who found the secretion in the anterior stomach neutral and in the posterior stomach weakly acid. Bellesme said that the secretion found in the stomach is acid and is derived from the coeca. Plateau in 1874 found the secretion slightly acid, but believed this to be accessory to the actual digestion, which he compared with pancreatic digestion. He removed the coeca and opened them on blue litmus powder. The powder was not affected, so he stated that the coecal secretion is alkaline. Krukenberg found the stomach secretion alkaline, while the acid secretion of the coeca flows into the crop to act on food there. More modern authors have uniformly found that the secretion of the stomach and coeca is acid in reaction.

In my investigations I first used the method of feeding a paste of sugar and litmus powder and dissecting the animals after various intervals. The contents of the coeca and anterior two-thirds of the stomach are always distinctly acid, while the contents of the hinder stomach may sometimes be alkaline. I have already given my reasons for believing that the acid secretion of the anterior stomach has its action not only in the stomach, but flows forward into the crop to aid in digestion there. This secretion is partly derived from the coeca and is no doubt very active in digesting all kinds of food, for the stomach is an important organ in secretion, digestion, and preparation of the food for the uses of the body. Schroder has proved that the coecal secretion has a digestive action on carbohydrates.

To make the matter of the reaction of the stomach more definite I removed the stomachs and coeca from ten animals, washed them in normal salt solution, and pressed out their contents. The ten stomachs and their coeca were ground with 10 cc. of salt solution and left for two hours. The mixture was then filtered and the filtrate titrated. The 10 cc. of the extract titrated against 0.3 cc. of 1/20 normal NaOH, thus indicating a distinct acidity. This shows that the digestive processes of the stomach occur in an acid medium.

The secretion, as Deegener and Jordan describe it, appears in little sacs which are formed at the ends of the cells and free themselves into the cavity. Holtz describes the pouring out of granules of secretion in *Nematus*. Steudel injected Congo red solution into the body cavity, and later found it appearing in more concentrated form in the extruded secretion sacs. Schroder holds that the sacs or lobes are not actual secretion, but that the secretion comes from a broader region of the cell. The projections may be for the secretion's dispersal, he says, and may form a broader surface for it.

VanGehuchten and Deegener have figured the release of spheres of secretion, and describe a cycle through which the secreting cells pass in three stages: 1) the formation of the sphere and its granular contents; 2) the completion of the sphere and its release; 3) the cell rests, meanwhile resembling a typical epithelial cell and probably being adapted to absorb soluble food substances. The process is described as continually going on, so that secretion is always ready for food when it arrives. The cells cannot form secretion spheres indefinitely long, but finally weaken and are cast loose with their last secretion sac. Several other authors have described the discharge of the oldest cells.

The appearances on my slides agree mostly with the observations of Deegener. The sacs at the ends of the cells and with their granular content are common, and many discharged sacs are seen in the lumen. Many regions of the epithelium show no signs of sac formation; these are in the resting stage. A density of the protoplasm near the ends of many cells indicates evidently a very early stage in the formation of a secretion of a secretion sac. All cells are able to secrete, provided they reach the surface, and the coecal cells show exactly similar sacs to those of the stomach, as we should expect from the similar histological structure. Both absorptive and non-absorptive cells are able to secrete. The absorbing cells are more active in secretion, perhaps because they are more mature. Every cell of a group of actively absorbing cells will very often show a large secretion sac. The presence of secretion is absolutely

necessary before fat can be digested and absorbed; in several cases I found the lumen full of fatty substance, but no secretion sacs in evidence, and therefore no absorption. This must mean that cells of a large region of epithelium undergo a resting stage at one time; the stomach may thus be actively secreting and absorbing while the coeca do neither, even if stuffed with food. Or the coeca may be active while the stomach is not. These observations give evidence that the stomach and coeca are similar in function, and that the coeca may be considered as expansions of the stomach which offer a large surface for digestive processes. It also seems clear that the enzyme which acts on fats in the stomach is entirely derived from the secretion globules of the cells.

Jordan and others have found a fat-splitting enzyme in the stomach. Plateau found an emulsifying power, and Jousset showed the presence of an acid after the enzyme's action. I have verified that the enzyme is a true lipase, which splits fats to fatty acids and glycerol. I will not here describe in detail my method of determining the lipolytic action of the stomach, for I used the same method as in the experiments on the crop. The stomachs and coeca of thirty cockroaches were extracted in 30 cc. of normal salt solution. Of the 30 cc. of the extract 10 cc. were left in a flask at room temperature for seventy-two hours; to another portion of 10 cc. was added 10 cc. of olive oil. The mixture was shaken and treated like the first. Both were titrated against $1/20$ normal NaOH. The portion without the olive oil titrated against 1.0 cc. of the alkali, while the portion with the olive oil titrated against 7.2 cc. of the alkali. This showed that the equivalent acidity of 6.2 cc. of alkali had been produced from the olive oil, and left no doubt that a lipolytic action had occurred and had produced the extra acidity through the formation of fatty acid as a digestion product of the olive oil.

The above results were obtained by using the stomachs and coeca together, so they did not make clear what separate parts the components took in the process. To determine this I dissected ten animals, removed the stomachs and coeca separately,

put them in separate dishes, washed in normal salt solution, and pressed out the contents. To the ten stomachs was added 10 cc. of normal salt solution and the same to the eighty coeca. They were ground to a pulp in separate mortars, left two hours for the extraction of the enzyme, then to each was added the same amount of olive oil, and they were left seventy-two hours and titrated. The dish containing the coeca showed more acidity than the stomachs in the ratio 7:5.

As the histological structures of the stomach and coeca are the same, a truer comparison of their lipolytic functions would be expressed in terms of equal areas of their epithelial surfaces. Examinations of freshly dissected and of sectioned material reveals that the coeca have on the average half the length of the stomach, and a third of its diameter. So each coecum has one-sixth the surface of the stomach, and the eight coeca have eight-sixths the area of the stomach. Thus the ratio of the epithelial areas of coeca and stomach is 8:6, and we have seen that the corresponding ratios of the lipolytic effects is 7:5. This close approximation is striking and makes it evident that the fat-splitting action of the epithelium of the stomach and coeca per unit of area (or volume) is the same. The coeca are therefore not the special organs of secretion which various authors have considered them.

The stomach and coeca are surely important organs for the secretion of digestive juices, at least as regards lipolytic action. Most authors have placed them first as secreting organs. They really occupy an important second place, the crop occupying first place and being about twice as potent in digestion, at least of fats, as the stomach and coeca combined.

Absorption in the stomach has been demonstrated by all investigators of insect digestion. All agree that the cells of the stomach have the power of absorbing food in liquid form. The older writers endeavored to demonstrate the passage of fat globules as such from the lumen into the cytoplasm of the cells, but the fallacy of this is evident since it is known that fats are split to soluble products before absorption. The descriptions of the process vary greatly as given by various investigators.

Among the earliest is that of Miall and Denny, who state in their text-book that the digestive products pass from the lumen through the protoplasm of the cells to the body cavity. We now know that the protoplasm has a selective absorptive action.

Petrunkevitch's opinion is, in brief, that in the stomach only the oldest cells, between the regenerating places, can absorb. Thus only a part of the inner surface of the stomach epithelium is capable of absorption. Digestion is considered as not very powerful here, but occurring mostly in the crop. The coeca are described as being structured similarly to the stomach and capable of absorption, but in less amount, for their function is mostly secretory.

VanGehuchten has described in one form a ring of special absorbing cells. This probably is not present in most insects.

Holtz has found that in *Nematus* the cells send out pseudopodia around the food and absorb it gradually. The pseudopodia are said to be distinct histologically and physiologically from the globules of secretion which are formed at the ends of the cells.

Cuénot found fat absorption in the posterior two-thirds of the stomach and in moderate amounts. The coeca are said to have a strong absorptive power. He thought the midgut was the only part of the alimentary canal capable of absorption.

Steudel in his work on cockroaches showed that only the non-secreting cells of the stomach were capable of absorption, and not the cells which were undergoing a process of formation of secretion globules. The unspecialized or resting stage is considered as an absorptive phase, which alternates in the cell with a secretory stage. He found conspicuous absorption of fat in the stomach and some in the coeca.

A great discussion has been waged over the question whether the stomach is the only organ capable of absorption. The absence here of the chitinous wall present in all other parts of the alimentary canal has led many on a priori grounds to believe that here and here only is absorption possible. Cuénot in 1896 stated that the stomach alone had the power of absorption while the chitin of all other parts was impenetrable. Kowal-

ewsky and Metelnikoff have used the method of pigment feeding, and conclude that almost all of the absorption occurs in the stomach. Metelnikoff fed food containing an iron salt; while it was being absorbed he precipitated the iron as Prussian blue: he found absorption in the stomach only.

Plateau, Petrunkevitch, and others, after thorough investigation, find the crop and important organ of digestion and the stomach of less significance. I have verified this, and further credence is given to this view by Sayce and others demonstrating that osmosis can occur through a chitinous intima.

My own investigations support those of Petrunkevitch. Fat is absorbed by the stomach cells and may be demonstrated in them by staining the tiny globules black by osmic acid. These globules first appear at very varying times after food is eaten, but no doubt only a comparatively short time after the food actually enters the stomach, provided secretion globules are present. Experiments with colored food have shown that fat may pass from the crop very soon into the stomach or may be entirely held back two or three days, even in animals which have been previously starved a long time. If the stomach is alone capable of absorption, as so many authors believe, it would indeed be hardly believable that an almost starved cockroach should be denied the power of absorbing or utilizing its food until several days after it has devoured it. What really happens is that the food begins to be absorbed and utilized by the crop soon after it is eaten.

The cells of the stomach are differentiated into older cells and regenerating ones; only the older cells can absorb. These usually occupy only a limited part of the surface of the epithelium, perhaps a third or less. The contrast between the absorbing cells and the others is striking, as shown in figure 21, where fat is being actively absorbed. Occasionally the upper ends of the neighboring cells may absorb a little fat. As this is only seen in the ends of the cells toward the lumen, it seems that fat is soon drawn from the neighboring cells through the lateral cell walls into the true absorbing cells. The cells which absorb most strongly secrete most strongly, as a rule, and there is almost no

absorption unless secretion globules from some cells are present to act on the fat. Rarely the epithelium is not specialized, but shows all cells alike. In this case fat globules may be found in the ends of all cells but even here there is a tendency for certain groups of cells to absorb most of the fat. I cannot support Cuénot in his statement that only the posterior two-thirds can absorb, for I find fat in the cells of the extreme anterior stomach. Serial sections of the whole length of the stomach show similar cellular structure throughout the entire length and similar absorptive power.

The coeca give the same pictures as the stomach. Figure 20 shows the arrangement in alternate groups of fat-absorbing cells and regenerating cells. I conclude that the coeca have approximately the same absorptive power per unit of surface as the stomach does. The fact that the coeca both secrete and absorb in just the same way as the stomach indicates that they are to be considered as proliferations from the stomach merely for the sake of exposing more stomach epithelium to food.

It does not seem to me at all probable that the limited amount of fat absorption observed in the stomach can be all the body is capable of. Only a part of the epithelium can absorb, and, moreover, more than half of my preparations of stomachs with fat in their lumina showed little or no absorption in the cells. This was due to lack of secretion or some other deterrent factor, and goes to show how irregular and comparatively slight is the absorption of fat in the stomach as compared with absorption in the crop, where I was practically always able to find good absorption stages at appropriate intervals after feeding.

The stomach of *Dytiscus* gives very similar pictures to that of the cockroach, though the coeca are small and numerous. A specimen was fed suet and dissected after three days. The stomach epithelium showed absorption of fat in groups of absorptive cells separated by regenerating cells, just as in the cockroach. The coeca also showed the same structure and absorptive power as the stomach.

In connection with the question of digestion in insects, a striking phenomenon has been described as occurring in the tracheae of the alimentary canal. This is the question of the presence of fats and other substances in the tracheal tubes after feeding, which has been under discussion more or less for about seventy years.

In 1847 Alessandrini noticed indigo in the tracheal tubes of moth larvae after they had eaten food containing indigo, both in the tracheal and cells and in the lumen of the tubes. Stimulated by this finding, Bassi did further work on the same material and concluded that Alessandrini's work was correct, but that only certain animals, and often only certain parts of these, showed the phenomenon, continuing to show it not only through the larval, but also the pupal and adult stages.

Blanchard injected pigments into the body cavity and found them later lying along the inner surfaces of the tracheal walls. He believed that they were carried there by the blood stream and named the process 'peritracheal circulation.' His results were quite uniform, and he considered the variability of Bassi's results to be due to variation in the amount of stained food previously eaten.

Soon after this Agassiz started his investigations on the problem and arrived at the conclusion that there are two sorts of tracheae, one respiratory and one circulatory, the two differing considerably histologically. These findings have not been substantiated by other authors.

Sadones found that there are, however, two distinct kinds of tracheal endings, one in the epithelium and one in the tissue below. Petrunkevitch has verified this.

Faussek described in certain insects tracheal tubes which enter the epithelium of the stomach and possess a distinct lumen bounded by plasma and normal nuclei, but no chitinous boundary. These were stated to end in sacs bounded by one or two nuclei. The appearances of sacs have since been criticized, and I agree with the critics that the sacs were probably artifacts caused by diffusion or currents. Free ending of tracheae were also described. This is in disagreement with the ideas of most investigators except Vieweger. The latter has described free intracel-

lular endings of tracheae in the cells of various tissues; these tracheae are said to branch in the plasma of the cells, some of the brachlets passing into adjacent cells. I have never been able to find any such striking appearances in my preparations.

Petrunkévitch described the end cells of the tracheae, and figured the two types which occur: one type forms an integral part of the epithelium of the crop and resembles the other cells except for its narrowness and the tracheal connection below; the other type lies immediately below the ordinary epithelial cells and does not extend to the intima (fig. 2). Within each type of cell may be found the minute ending of the lumen of a tracheal branchlet. The tracheal tubes are bounded throughout by peritracheal cells, in which nuclei are always visible, but whose cell boundaries are not defined.

Petrunkévitch has also described four very interesting appearances which occurred in preparations made at intervals after fat had been fed. These four phenomena are: 1) the presence of many tiny globules of osmicated fat in those tracheal end cells which form an integral part of the epithelium, the globules being identical with those of the ordinary cells, and evidently representing fat absorbed from the lumen; 2) fat in the tracheal tubes, usually lying along the inner walls of the tubes and upon the coils of the spiral, supporting structures, and even moving along the spirals or moving out into the lumen to lie in a sticky deposit there; 3) the presence of globules of osmicated fat in the peritracheal cells; 4) the presence in the lumen of certain tubes of a sticky deposit which resembled the contents of the crop's lumen, and which sometimes contained leucocytes and fat globules. These four appearances were described as representing four stages in one process which occurred as follows: The fat is absorbed by the tracheal end cells and passes from their cytoplasm into the minute terminal lumina of the tracheal tubes. In these the fat tends to lie on the spirals, and to proceed along the tubes in a spiral course by slipping along the spirals. It is now absorbed by the peritracheal cells and utilized by them. Not only fats are passed into the tracheal tubes through the tracheal end cells, but also other foods, and these appear as a

sticky mass in the lumen. This mass was called chyme; it often contained cells which exactly resembled leucocytes. These cells seem to devour fat and other foods.

Schlüter employed methods somewhat similar to those of Petrunkevitch, but has disagreed with him in almost all the details described. He is unable to find any evidence of absorbed fat in the epithelial or tracheal end cells. I have carefully examined the data of his experiments which he has published, and I find that the time intervals he used were so chosen that only in a very few cases would absorption probably be in evidence. In only two of his experiments as given would significant absorption be expected, judging from my results. These two experiments apparently represented single animals, so it seems that the chance individual variation of a very few animals from the usual absorptive function really explains Schlüter's denial of absorption in the epithelial and tracheal end cells.

Schlüter assumed that appearances of fat and chyme in tracheal tubes indicated a chance pushing or slipping of substance from other regions into the tracheae during sectioning of the material or preparation of the slide. Or the contents of the tubes may have entered them by capillary action during dissection. Schlüter's final objection is that as such appearances have no reason nor physiological explanation, they have no actuality.

In my first experiments I found stages which corresponded very closely with those of Petrunkevitch. I used animals which had been starved two weeks and fed them a paste of olive oil and pulverized sugar. The animals were removed after certain intervals and dissected, immersed in normal salt solution. The crop was slit longitudinally so that the contained food might float out, the tracheal trunks of the crop were cut and the crop was removed and placed in Flemming's fluid. Flat mounts of the wall of the crop were made and also paraffin sections. Such preparations revealed the presence of black fat in the tracheal tubes, either in some of the smaller tracheae or in both large and small tracheae. The pictures corresponded so closely with those of Petrunkevitch that I believed I had verified his work, and I so stated in a preliminary paper.

In attempting to analyze the explanation and significance of the intratracheal fat, I soon found that if I bathed a cockroach in olive oil, the oil entered the spiracles readily and filled some of the tracheal tubes to a considerable extent. Schlüter has suggested, too, that some fat might enter the spiracles. This showed plainly that in work with tracheal tubes a new factor must be considered, namely, the sucking action of the tubes due to negative pressure. This factor was not used or eliminated in experiments by previous authors, and I now realized that I must always consider it in further experiments.

Moreover, the fact that olive oil enters the spiracles affords an entirely new method of attacking the problem. The oil enters during the bathing process because it is sucked in by the respiratory movements which cause negative pressure within; oil is sucked in until pressure is equalized throughout the tubes, so that all air which is contained in the tubes is now at atmospheric pressure. The animal may now be bathed in the oil until it dies through suffocation due to blocking of the spiracles and therefore its breathing movements cease. Complete equalization of pressures throughout the tracheal system is then assured, and consequently there can be no subsequent sucking action. Knowledge of this process allows a new method of investigating whether or not fat is normally present in the tracheae after stained fat has been eaten. For an animal can be bathed in oil which contains a stain of contrasted color to that of the stain which the food contained, this differently stained oil used for bathing will then enter the spiracles and equalize the pressures in the tubes, so all possible danger of sucking action in them during subsequent dissection is obviated. As red oil was used in feeding, green oil would be ideal for the bathing process. However, no brilliant green stain for fat was available, so I used a green solution which was found to enter the spiracles and tubes just as oil did. This was a mixture of equal parts of glycerin and 70 per cent alcohol, and saturated with brilliant aniline green. By using this mixture to bathe until they died animals which had previously eaten red fat, it is evident that the following set of conditions would be given: green fluid in the main trunks, pre-

venting entrance of any other substance from without; air in the remainder of the tubes at normal pressure, so there is no danger of sucking action anywhere even if tubes are injured or cut; red, if any, in the tubes, indicating a normal content of red fat in the tracheae due to metabolic processes and by no chance to the conditions of the experiment. The actual set of experiments is the following:

One hundred and twenty-six cockroaches were fed a paste containing powdered sugar, olive oil, and Sudan III, these were removed at intervals. Eight hours after feeding three of them were removed from the jar, their necks were tightly ligated with silk to prevent any fluid used in the experiment from being swallowed, and they were immersed in the green mixture of glycerin and alcohol. After a short period of extreme activity, the animals died by suffocation, due to the liquid entering the breathing tubes; they were then removed from the green solution, washed, and dissected. It was found that the green had penetrated a considerable distance into the tracheal trunks, it had been drawn in by the sucking force present in the tubes before the animal died. The presence of this green fluid in the tracheae during the dissection prevented the entrance of any other substance into the tubes, not only by way of the spiracles, but at any place, for pressures were equalized. The trunks were cut in the region containing the green and the alimentary canal was carefully removed without injuring the wall of the crop. It was mounted in toto in glycerin and examined microscopically for the presence of any red substance in the tubes; such red substance would if present mean that red fat had been derived normally from the food in the crop's lumen. But no red fat could be detected in the tubes.

Three more of the animals were removed and treated similarly sixteen hours after feeding, three more at twenty-four hours, and three more at the end of eight eight-hour period throughout two weeks. In none of the 126 animal used could distinct redness be observed in the tracheae. Other animals were fed and dissected under the same conditions except for the fact that the crop was slit longitudinally during dissection; these showed no red sub-

stance in their tracheae, nor did a series of animals which were treated similarly but for the fact that their crops were preserved in Flemming and sectioned; microscopic examination of sections of this material revealed no osmicated fat in the tracheae.

The experiments had comprised all usual digestive periods and had included so many animals that individual variation and other complicating factors were negligible. The result was considered final and definite; it was certain that fat did not normally enter the tracheal tubes, as I had previously thought. I realized that all the fat I had seen in tracheae in earlier preparations must have come from an abnormal source.

I next attempted to analyze why my earlier experiments, like Petrunkevitch's, showed fat in the tubes, while more careful controls did not. From previous observations with colored fat I knew that oil might enter the cut ends of large tracheal trunks, but obviously this did not explain why Petrunkevitch and I had found fat present in only certain small branches and groups of branches. Another factor had obviously been acting. In attempting to explain this I performed the following experiment: An animal which had eaten some red paste two days before was pinned, while still active, dorsal side down on a dissecting pan. The spiracles were then thickly smeared with celloidin solution. This soon dried and formed a complete film over all the spiracles, thus preventing the entrance of any substance into the tracheal trunks within. The passage of air was prevented, but the still living animal performed to some extent breathing movements. The result of these movements is suction, but nothing could be sucked in, so the suction existed in the tubes without any possibility of anything being sucked in. The animal's body wall was carefully slit and pinned to the side without disturbing the tracheae. Now the wall of the crop was slit longitudinally. This necessarily involved cutting many of the tracheal branches of various sizes which ramify over the crop. As soon as the animal was surely dead, the crop was removed and its outer surface examined microscopically. The small tracheae which had been unavoidably cut were especially observed, and it was found that some of them contained red fat

in the region of the cut ends and for a distance beyond. This surely did not represent a normal process, but resulted from some factor which had been acting through the experiment. It seems certain that the negative pressure in the tracheae, as mentioned above, had exerted a sucking action at points where openings were made. Such openings were made when the crop was slit longitudinally, and as a result the substance at the cut end (in this case olive oil) had been drawn in. This experiment was repeated on other animals and showed conclusively that the fat which Petrunkevitch and I had found in minute tracheae in our preparations had been sucked in by the tubes through their cut ends during the dissection, and was therefore no normal content, but an artifact. We had easily overlooked it because such small structures were involved and because we had used colorless fats. It was interesting to explain why Petrunkevitch found fat in the tubes only at considerable intervals after feeding, while I found it at many stages. This is simply due to the fact that Petrunkevitch fed solid fat which required some time to be liquefied and changed to fatty acid and thus made capable of being sucked into tubes. In my work, liquid fat or fatty acid was always present in the crop after feeding, and it was thus always possible that it be drawn into the tracheal branches by suction, no matter at what period after feeding the cut was made.

The above experiments demonstrate that fat in tracheal tubes is abnormal; its presence may be accounted for in three ways: 1) Suction of fat through minute tracheae which are cut when the crop is opened in dissection; this suction may occur centrifugally or centripetally; 2) suction through large tracheal trunks and through spiracles; 3) capillarity. These three factors seem sufficient to explain the pictures of the several investigators who have so long discussed the problem; they indicate that fat is never normally present in the tracheal tubes.

The other three appearances which Petrunkevitch described and figured must now be explained, and, firstly, the globules of osmicated fat in the tracheal end cells. I have verified this in various preparations. These cells resemble in structure the epithelial cells, so it is natural that they absorb soluble food products

in the same way. It cannot be definitely stated whether the fat gets merely into the end cells or also into the first of the peritracheal cells, for cell walls are indistinguishable. I do not believe that fat ever gets from the cytoplasm of the tracheal cells into the lumen of any tracheae.

The fat globules found in the peritracheal cells are easily explained. They only appear occasionally in my slides, for the cytoplasmic layer is very thin in the American cockroach (fig. 14), and not of such thickness as in the oriental cockroach. The peritracheal cells are bathed by the blood which fills the body cavity, and they take from the blood the food products which they need. The blood contains much fatty substance after fat has been eaten, and this substance may readily be absorbed by the peritracheal cells and reorganized to fat, which appears in globules in the cytoplasm. Similar globules may also be seen in the cells of the Malphigian tubules, which are similarly bathed by the blood.

The last phenomenon described by Petrunkevitch is the presence of chyme and leucocytes in the tubes. I have found such chyme present in practically all my preparations of the crop, and leucocytes were often present in it (fig. 14). It was present even in the most careful controls, where the animals were immersed in the green solution before dissection; it usually appeared in some of the smaller tracheae, but sometimes in the larger ones. There is no doubt that the presence of a sticky deposit is a common occurrence in some tracheae of American cockroaches. Its origin is very difficult to explain. In view of experiments already described, it is certain that it does not enter the tubes through the tracheal end cells. My explanation is only tentative and cannot be tested experimentally nor verified by observation. It is well known that many Orthoptera have difficulty in moulting, often dying or sustaining injuries in the process. It seems possible that during the moulting of cockroaches slight disturbances might cause rips or breaks in the tracheae. This would be fatal if it occurred much in the larger tracheae, but might cause little inconvenience if it happened in the smaller branches. Blood would then fill the torn branches with its con-

tained leucocytes. The deposit in the tracheae is always much thicker than blood; it may be that some of the water diffuses out of the blood in the tubes. Perhaps the leucocytes ingest some of the unnatural substance present. The following experiment was performed to determine whether leucocytes might enter tracheal tubes which contained an unnatural substance.

A normal animal was immersed in olive oil which was stained red. The animal was dissected, and the tracheal trunks were found to contain much red fat. A piece of one, filled with fat, was removed and inserted into the body cavity of another normal animal. This animal was dissected three days later; the red trachea was removed, preserved in Flemming and sectioned. The tube was found to contain many leucocytes which had entered in response to the unnatural substance, and were ingesting it, as evidenced by the globules of ingested fat in their cytoplasm. The result of the experiment suggests that leucocytes may enter any rips which occur in the tracheae and may ingest any foreign material there, whether fat, thickened blood plasma or other substances. Such a theory is incapable of proof. It is almost certain in any case that the chyme is not to be considered as a really normal content.

My investigations have shown that three of Petrunkevitch's four striking appearances represent usual conditions, his slides agreeing closely with mine. Therefore Schlüter's absolute and critical denial of his observations is unjustified. As to the interpretation of these four observations, I am convinced that they represent four distinct processes, two of them normal, and not four stages in one process, as Petrunkevitch thought.

Blanchard's 'peritracheal circulation' theory should be considered here. In order to test whether substances really can pass from the blood into the tracheae, as Blanchard described, various substances were injected into the body cavity and later searched for in the tracheae. Such injections had little or no toxic effect. The best substance to use is phenol-sulphone-phthalein, which passes through the body dissolved in the body fluids. The presence of this stain could not be detected in the tubes at any of several periods after injection, though the stain

was absorbed by the stomach and intestine. Perhaps Blanchard's conclusions of 1849 may be due to poor definition of tracheal lumen and peritracheal cells on his slides. My experiments support Petrunkevitch in stating that there is no evident peritracheal circulation in the cockroach.

In conclusion, I wish to thank Professor Petrunkevitch, under whose direction the work was done. I wish, too, to thank Professor Mendel for his advice concerning some of the experiments, and Professors Osborne and Mendel for some of the reagents used; also Mr. W. S. Sturges for assistance with the titrations.

SUMMARY

1. The crop is the principal organ of digestion in the cockroach. Its enzyme splits fats to soluble products. The progress of digestion may be observed by giving food containing indicator stains. Digestions may be carried on outside of the body, and quantitative estimations of the amount of digestion may be made by titration. Such tests show that the crop is at least three times as active as the stomach in fat digestion.

2. The crop is the principal organ of absorption of fats. The process is rather slow and reaches a maximum two days after the fat is eaten. The absorption of one large meal may take two months. All cells of the epithelium of the crop can absorb; the cells may store globules of fat until it is needed.

3. The crop is an important organ for storage of food; it may store enough to supply the needs of the animal more than two months.

4. The gizzard has an important sphincter action, and may withhold food from the stomach several days. The sphincter action resides in the folds which protect the passage from the gizzard into the stomach.

5. The needles on the six significant folds or cushions of the posterior region of the gizzard are moved by special muscle fibrils and tendons derived from long strands of striated muscle. The function is difficult to ascertain, but possibly they aid in moving food through the gizzard.

6. The coecal epithelium has the same function and histological structure as that of the stomach. It exhibits somewhat more surface than the stomach, so that the digestive (enzymatic) power is somewhat greater than that of the stomach. The secretion appears as little spherical sacs released from the cells.

7. The stomach and coeca absorb food in groups of absorbing cells. These groups are separated by groups of undifferentiated and immature cells which will later take the place of the older ones as they are cast at intervals.

8. The tracheal end cells absorb fatty products from the lumen of the crop, and the peritracheal cells absorb them from the blood. The tracheae themselves never contain fat normally. The presence of fat and other substances in the lumina is due to imperfect methods of preparation.

9. Certain tracheae of practically all animals contain a sticky substance whose origin is uncertain. Leucocytes are often present in this substance.

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PLATES

ABBREVIATIONS

| | |
|--|--|
| <i>AE</i> , group of absorptive cells of stomach | <i>MB</i> , fibers of one of the six muscle bands |
| <i>AM</i> , anterior muscle sphincter of gizzard | <i>MF</i> , fibrils derived from fibers of one of the six muscle bands |
| <i>CA</i> , group of absorbing cells of caecum containing absorbed fat | <i>MT</i> , tendons derived from muscle bands |
| <i>CC</i> , cells of epithelium of cushion | <i>NE</i> , needles |
| <i>CE</i> , epithelium of caecum | <i>PC</i> , peritracheal cell |
| <i>CH</i> , chitinous pieces in lumen of stomach | <i>PM</i> , posterior muscle sphincter of gizzard |
| <i>CU</i> , cushion | <i>PS</i> , passage from gizzard to stomach |
| <i>DC</i> , deposit in tracheal tube | <i>PT</i> , primary teeth |
| <i>EC</i> , epithelium of crop | <i>RF</i> , radial muscle of crop |
| <i>FC</i> , muscle fibers of crop's wall | <i>ST</i> , secondary teeth |
| <i>IC</i> , intima of cushion | <i>TT</i> , tertiary teeth |
| <i>IE</i> , intermediate cells of stomach epithelium | <i>TC</i> , tracheal end cell |
| <i>LC</i> , leucocyte | <i>YC</i> , young and embryonic cells of stomach epithelium |

PLATE 1

EXPLANATION OF FIGURES

1 Section of wall of oesophagus, showing the processes of its surface, the intima, the cells, and a few muscle fibers. Flemming. Acid fuchsin. 525 diam.

2 Transverse section through the wall of the crop, showing the cells and the intima, the ends of tracheae and the attachment of a radial muscle to the intima. Flemming. Acid fuchsin. 325 diam.

3 Transverse section through wall of the crop, showing the result of absorption of fatty material thirty-six hours after ingestion of olive oil. The cells contain many globules of osmicated fat. Flemming. Acid fuchsin. 325 diam.

4 A later stage of the same process. The cells contain a maximum amount of absorbed fat. Flemming. Acid fuchsin. 325 diam.

5 Transverse section of crop of animal which had eaten a large meal of olive oil two months previous to fixation; no other food was given. The cells contain globules of stored fat, which are being gradually broken down and utilized. Flemming. Acid fuchsin. 325 diam.

6 Section through wall of crop, showing the result of absorption of oleic acid thirty-two hours after ingestion. Flemming. Acid fuchsin. 325 diam.

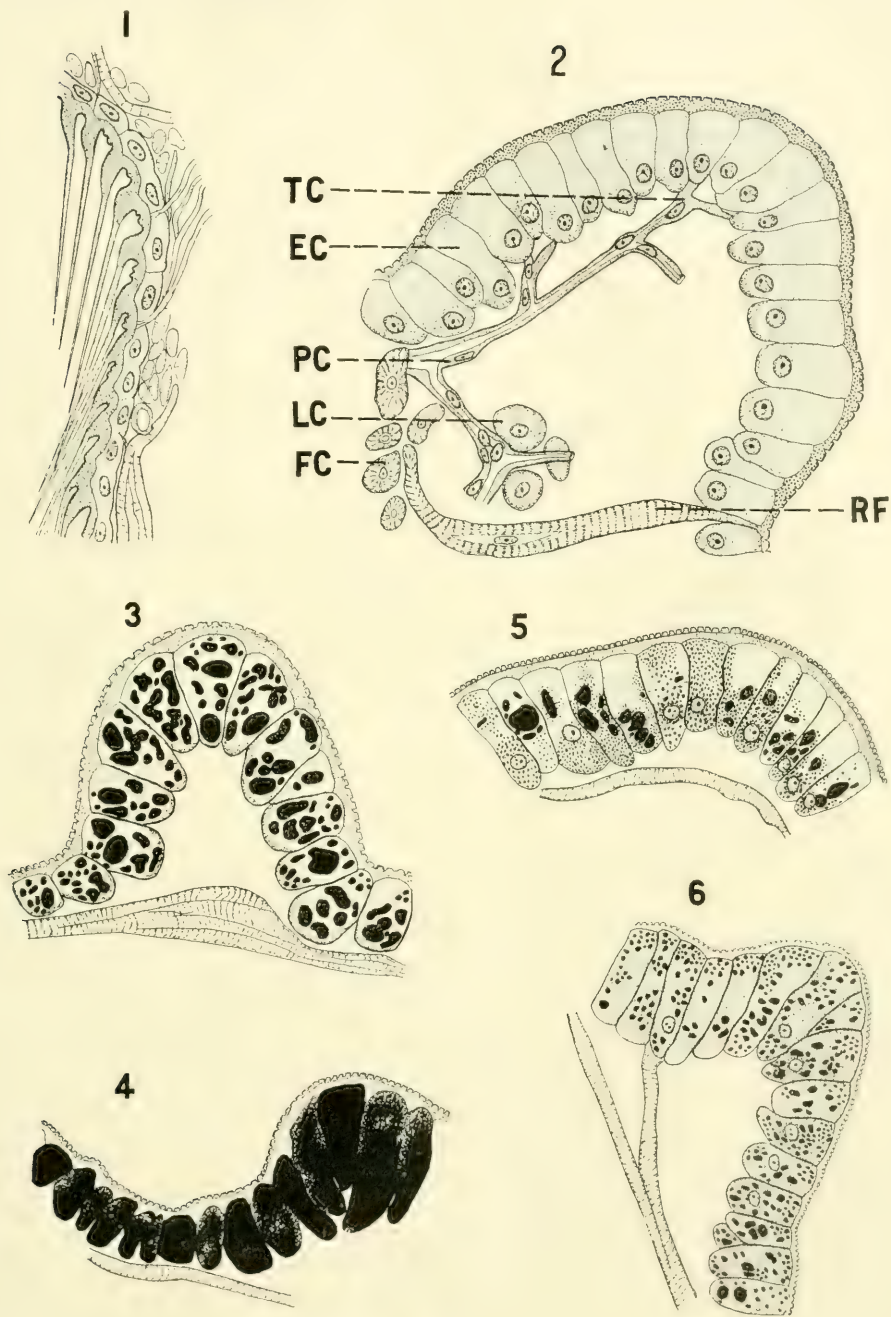


PLATE 2

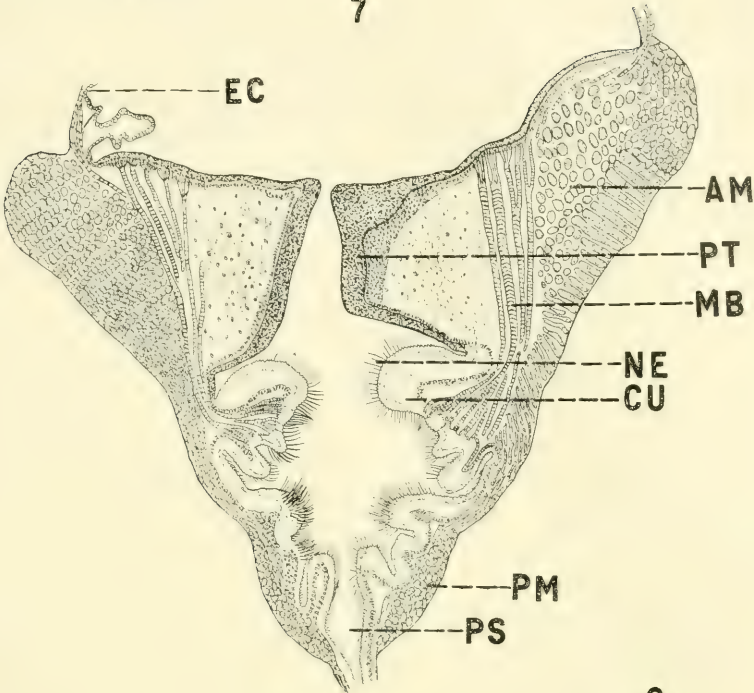
EXPLANATION OF FIGURES

7 Longitudinal median section of gizzard, showing the heavy teeth, the cushions behind them, the folds of the posterior part, and the passage to the stomach; also the circular sphincter muscles of the anterior and posterior regions and two of the longitudinal muscle bands. Flemming. Iron haematoxylin and picro-fuchsin. 35 diam.

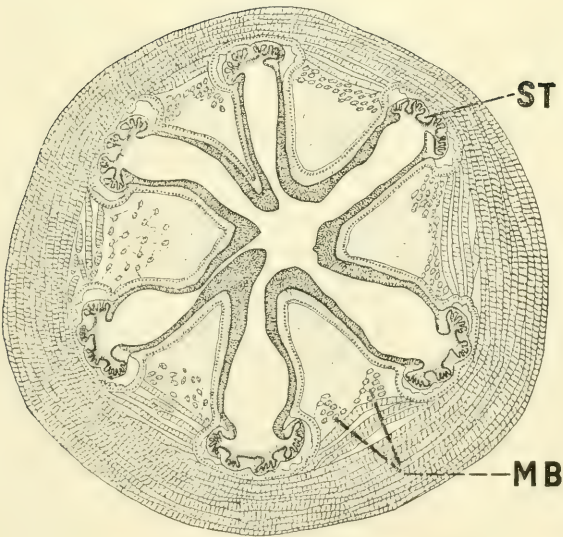
8 Transverse section of anterior gizzard, showing the teeth and the surrounding circular muscles, also the six longitudinal muscle bands in cross section. For details of the region between the teeth see figure 9. Flemming. Acid fuchsin. 35 diam.

9 Transverse section of the secondary and tertiary teeth of the anterior gizzard; showing also the epithelium and circular muscles. Flemming. Acid fuchsin. 90 diam.

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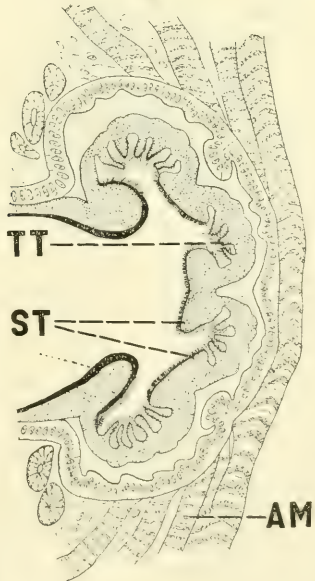


PLATE 3

EXPLANATION OF FIGURES

10 Transverse section of the gizzard in the region of the six cushions, showing the passage of the lower ends of the six muscle bands through the cushions to the needles on their surfaces. Flemming. Iron haematoxylin and picro-fuchsin. 35 diam.

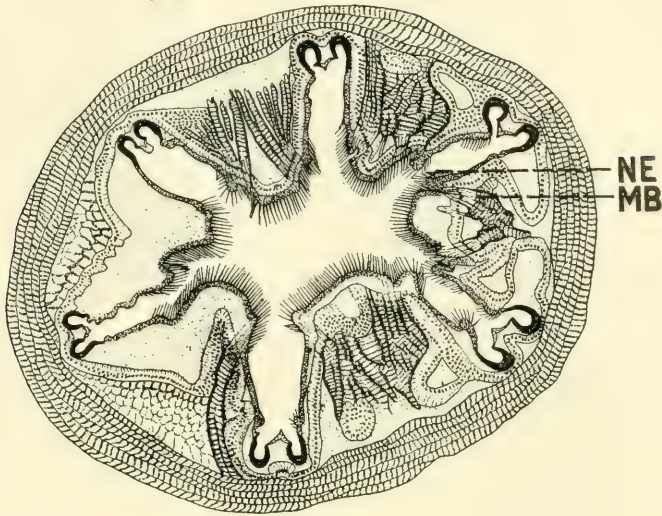
11 Detail of the connection between the muscles and the needles of the cushions. It is shown that the striated fibers divide to fibrils, themselves striated. These pass through the epithelium and become tendinous. The tendons cross the matrix of the intima and insert in the bases of the needles. Flemming. Iron haematoxylin and picro-fuchsin. 200 diam.

12 Section through intima of a cushion, nearly parallel to surface, showing in transverse and diagonal section the tendons which traverse the intima. Zenker. Iron haematoxylin and picro-fuchsin. 90 diam.

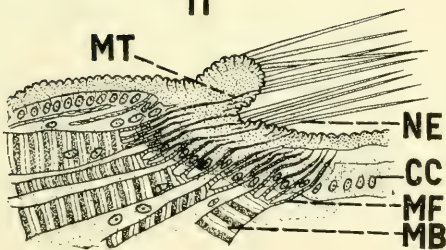
13 Surface view of a group of bristles of the cushions, showing the chitinous projections to which they are movably attached. 200 diam.

14 Section through a tracheal tube which is filled with coagulated deposit, in which leucocytes may be seen. Flemming. Acid fuchsin. 350 diam.

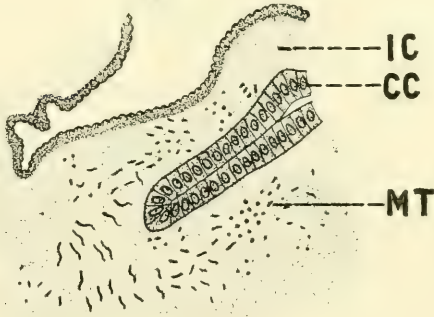
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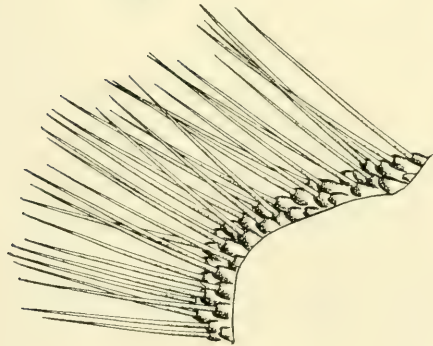
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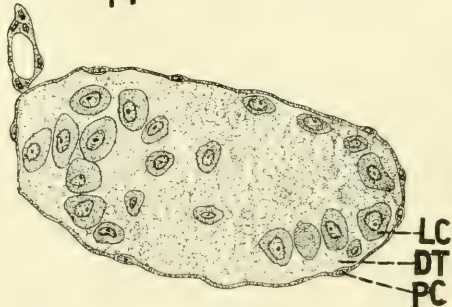


PLATE 4

EXPLANATION OF FIGURES

15 Longitudinal section through hinder end of the gizzard, showing the closely appressed folds of the inner wall and the thick surrounding sphincter muscle, which seems to make a tight closure. Flemming. Acid fuchsin. 35 diam.

16 Transverse section of the same region, showing the folds almost closing the passage, and the surrounding sphincter. Flemming. Acid fuchsin. 60 diam.

17 Transverse section of the wall of the crop, showing in one place an abnormal appearance or 'lesion' of the epithelium. Flemming. Acid fuchsin. 325 diam.

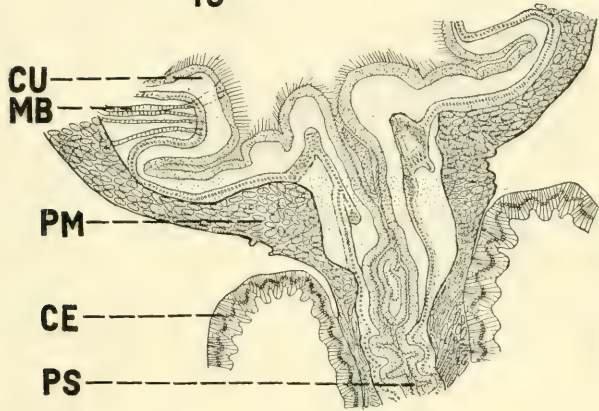
18 Surface view of epithelium of the crop, showing unusual conditions. Several binucleate cells are present, and also a region of cytoplasm where cell walls are lacking. Perenyi. Ehrlich's haematoxylin. 200 diam.

19 Transverse section of stomach two days after ingestion of fat. The epithelium shows frequent dark regions which indicate absorption of fat here. The lumen of the stomach contains oil drops, pieces of chitin, and other substances. Flemming. Acid fuchsin. 35 diam.

20 Transverse section of a caecum, prepared in the same way as the stomach of figure 19, and showing a similar condition of fat absorption. 35 diam.

21 Transverse section of epithelium of stomach's wall after ingestion of fat, showing in more detail the groups of cells which absorb fat, and also the embryonic cells which continually make new cells to replace the lost ones. Flemming. Acid fuchsin. 200 diam.

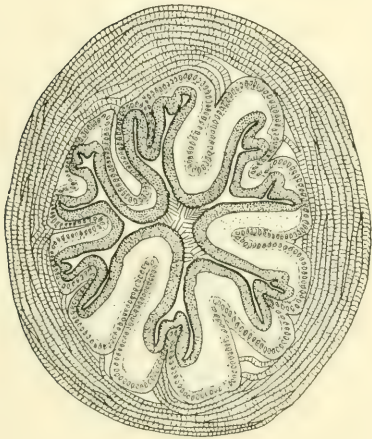
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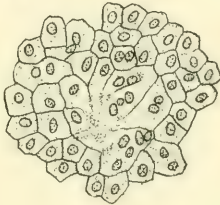
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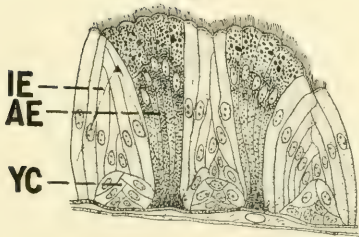
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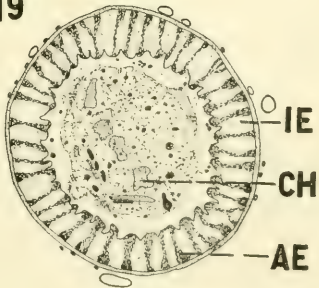
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19



EXPERIMENTS ON THE DEVELOPMENT OF THE FORE LIMB OF AMBLYSTOMA, A SELF-DIFFER- ENTIATING EQUIPOTENTIAL SYSTEM

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FORTY-FIVE FIGURES

Since Balfour's ('78) discovery of muscle buds in the developing fins of elasmobranchs the composite nature of the vertebrate limb has been emphasized. In the lower forms at least five different embryonal tissues are involved in its make-up, and these recur in part metamerically. Primarily there is a local thickening of the somatopleure which gives rise to the main bulk of the limb—the skeleton and other connective-tissue elements. This is covered over by ectoderm which in that region shows distinct modification. Into the mesenchymal mass derived from the somatopleure the muscle buds, blood vessels and nerves grow sooner or later from the trunk of the embryo.

Some years ago it was pointed out (Harrison, '95) that the various fins of the salmon form a distinct series as regards original visible complexity, beginning with the median fins where metameric muscle buds are clearly formed, as in the elasmobranchs, and ending with the pectoral fin, which receives no muscle buds. Here the proliferation of the somatopleure forms a blastema which gives rise to the muscles as well as to the skeletal elements.¹ As compared with the primitive elasmobranch fin, the pectoral fin is thus a more compact and unified organ at the time of its origin, and this is true in even higher degree for both extremities in the Amphibia. Probably also the limbs of the

¹ Derjugin ('08) has described what purport to be muscle buds in the pectoral fins of *Exocoetus volitans*. In view of the differences in the fins of a single species, *Salmo salar*, it would not be strange, were specific differences of this kind to be found in the pectoral fin of various bony fishes.

Amniota, with possible exceptions among the Reptilia (van Bemmelen, '89, Mollier, '95) develop according to the latter type.

The first experimental work aiming at an analysis of these factors was done by Byrnes ('98 a), who found that the normal development of the posterior extremity in both *Amblystoma* and *Rana* is independent of the presence of the muscle plates of the limb region. More recently this same point has been demonstrated with greater precision by Lewis ('10), who has shown that while extirpation of muscle plates in the limb region of *Amblystoma* produces very definite defects in the lateral and ventral musculature, it has no effect upon the development of the limb itself.

It was found by Langnecker (Harrison, '04) that the development of the skeletal and muscular elements of the limbs of the frog would take place even when the nerves of the limb were excluded by operation on the embryo. This was afterwards confirmed by Braus ('05), using different methods of experimentation.²

There remain, then, as possible factors determining the differentiation of the limb bud from the body wall in general, *a*) the potencies of the ectoderm cells of the region; *b*) the potencies of the somatopleuric mesoderm cells, and *c*) the position of these elements with respect to the organism as a whole, or perhaps better with respect to its immediate surroundings, including, in the case of the fore limb, the pronephros and the branchial region,³ which have close topographical relations to the limb rudiment. The blood vessels are not included in this enumeration, for while it is obvious that they are necessary for the nourishment and growth of the limb, no specific action on their part can be assumed. Braus ('03, '04, '09) found that the limb bud of the anuran larva constitutes a self-differentiating system

² Braus ('06 a) also showed that in the elasmobranch fin certain skeletal elements, the cartilaginous rays, would develop independently of the muscle buds.

³ Braus has suggested ('06 b, p. 545), on the basis of experiments upon *Rana* embryos, that the gill region may exert a formative influence upon the regeneration of the fore limb, though no evidence is adduced to show whether this region has an influence upon the normal development of the limb.

which develops into a normal limb when transplanted to new and strange surroundings. This important discovery, which was confirmed independently by Banchi ('04, '05) and later by the present writer ('07) shows that once the development of the limb has started, its position in the organism as a whole does not affect its specific character.

The experiments taken up in the present paper, which deal with the fore limb of the urodele embryo, are designed to apply the tests of self-differentiation to a much earlier period of development. Considering for the present only those stages with closed medullary folds, the experiments show that the immediate organic environment of the limb rudiment, even before visible differentiation sets in, has no specific influence in determining that a limb shall develop, though it may affect the posture and laterality of the limb that does arise (Harrison, '15, '17). The essential process of differentiation, whereby the potency to form a fore limb becomes localized in certain cells of the body wall, must therefore be relegated to very early embryonic life.⁴ It is further shown that in the stages under consideration this potency is localized in the mesoderm of the region involved, the ectoderm remaining indifferent.

The object of the experiments, aside from their purpose in further analyzing the factors taking part in the formation of the vertebrate limb, was to study certain relations which have a direct practical bearing upon the experiments on the laterality of limbs to be described later. An effort has been made to determine precisely the extent of the limb rudiment in the stages used for operation. In addition, the question of the equipotentiality of the elements composing the limb rudiment has been investigated both by defect experiments and by transplantation.

In interpreting defect experiments there is always a serious source of error in the occurrence of processes of regeneration or regulation, whereby elements which do not normally give rise to the part which has been removed may do so vicariously under the changed conditions. Such processes are known to occur in connection with experiments upon the limbs and must

⁴ Before the formation of the medullary folds according to Detwiler ('18).

therefore be taken into consideration. For instance, Miss Byrnes ('98 b), working with *Rana* embryos, found that if the region in which the hind limb develops is destroyed, a limb nevertheless develops out of the tissues that move in from the periphery and cover the wound. Braus ('06 b) found, however, that in *Bombinator*, as well as in *Rana*, the hind-limb region is much more capable of this kind of regeneration than is the fore-limb region.⁵ In the case of the latter, as Braus ('09) shows, it is only the shoulder girdle and some of the muscles that are formed after removal of the limb bud, unless the operation is done at a comparatively early stage, in which case regeneration of the whole extremity may take place. When the limb bud is itself transplanted it gives rise to a small shoulder girdle with some of the muscles in addition to the free appendage, so that in such an experiment certain muscles and skeletal elements develop only at the seat of origin of the bud, certain others develop only out of the transplanted bud itself, while again others develop in both places. The same has been found true in many cases in the course of the present work upon *Amblystoma*. Under certain conditions, however, the whole limb may be regenerated after removal of the bud, two complete limbs being thus derived from the single limb rudiment. The size of the extirpated region of body wall and the completeness with which all mesoderm cells within that region are removed have been found to be important factors in determining whether complete regeneration takes place or not.

In connection with the duplicated development of the shoulder girdle in its original position and at the seat of implantation, Braus has treated in full the question of mosaic development vs. equipotentiality.⁶ He reaches the conclusion that in the *Anura* the shoulder girdle constitutes an equipotential restitution system, though in its original surroundings there is evidence of localization of materials before visible differentiation sets in. The results recorded in the present paper agree as to the main

⁵ In *R. esculenta*, according to Braus ('09, footnote, p. 194), the power of regeneration is greater than in *Bombinator*.

⁶ Braus, '09, p. 271, et seq.

facts with those of Braus, though they leave uncertainty as to whether in *Amblystoma* there is complete qualitative restitution of the shoulder girdle in either place.⁷ Whichever view may be accepted for the shoulder girdle, all of the evidence accumulated here goes to show that the free extremity, while self-differentiating, must be considered as an equipotential system in Driesch's sense.⁸

MATERIAL, METHODS AND NORMAL DEVELOPMENT

All the experiments have been made upon embryos of *Amblystoma punctatum* in stages ranging between those shown in figures 1 and 3. This species offers great advantages over the anurans, which have been largely used in previous experiments upon the limbs. The fore-limb rudiment develops very early and rapidly, and may be located with precision shortly after the medullary folds close, before the embryo can move, and before blood vessels or nerves have developed. Experiments can, therefore, be carried out without anaesthesia, and the different tissues or embryonic organs can be easily separated from one another. The absence of a closed operculum, which in the anurans covers up the fore limb, is also a great advantage in that it permits the study of the progress of the experiment on the living individual, which may be examined from day to day without difficulty.

The technique of the surgery of the Amphibian embryo is now so well known that no general description is required here. The special operations used in the present investigation will be described as far as needed in the separate sections.

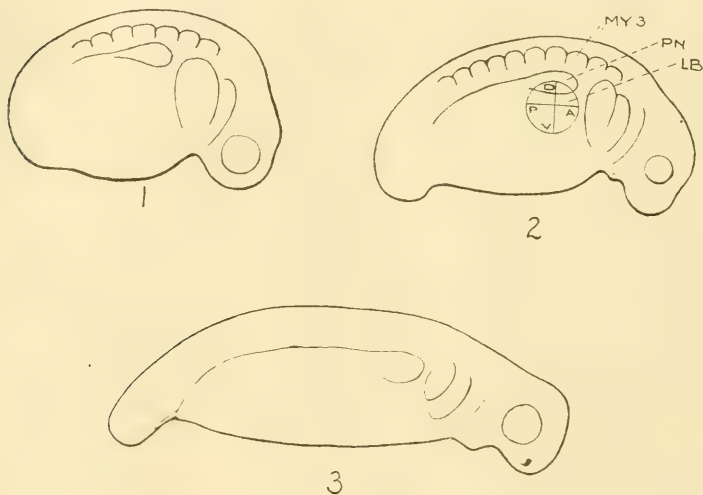
In the earliest stages at which operations were done the limb rudiment cannot be distinguished by any definite characteristic save its location. In the stage with beginning tail bud (fig. 1, stage 25⁹) the pronephric swelling becomes visible and the muscle

⁷ A more complete analysis of the case is given by Detwiler ('18).

⁸ Driesch, '99, '05, p. 679.

⁹ In the absence of a set of 'normal plates' of *Amblystoma*, a series of stages have been designated arbitrarily and type specimens preserved. In course of time, if the necessary drawings can be prepared, it is hoped that they may be published.

plates may be observed through the skin. Though there is no distinct limb bud present, the region centering in the fourth segment just ventral to the pronephros contains the material that will give rise to the limb. A little later, in the standard operating stage when the tail bud is more marked (fig. 2, stage 29), sections show that the somatopleure ventral to the pronephros is thickened. Still we can scarcely speak of a 'limb bud' on the



Figs. 1 to 3 Embryos of *Amblystoma punctatum* in the stages used for operation. $\times 10$. A, D, P, V, mark the four cardinal points (anterior, dorsal, posterior, ventral) of the limb bud (LB); MY3, third myotome; PN, pronephros. Figure 1. Earliest stage (stage 25). Figure 2. Standard operating stage (stage 29). Figure 3. Oldest stage (stage 33).

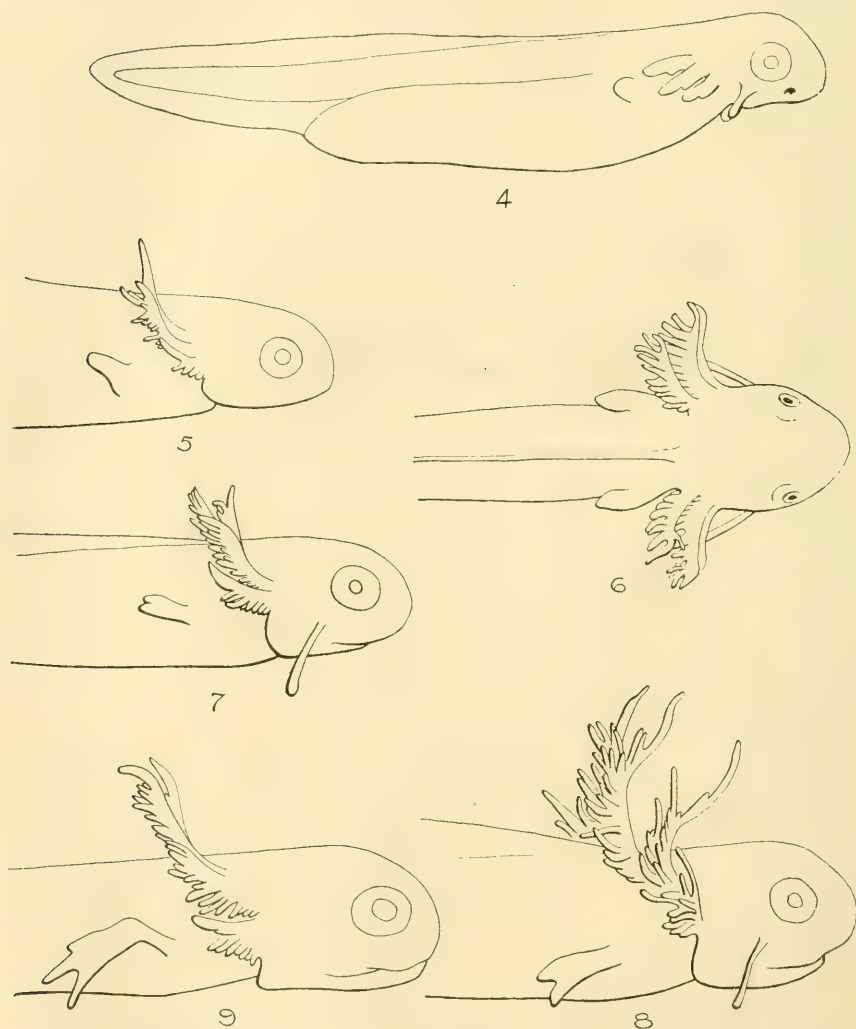
surface of the embryo, for the pronephric tubules are the chief cause of the swelling in that region, the somatopleural thickening merely serving to round it out on the ventral side. After this period the somatopleural proliferation increases, making the prominence on the side of the embryo more distinct (fig. 3, stage 33), but it is not until several days later that the extremity itself appears on the surface as a more sharp elevation in the

region of the fourth somite ventral and somewhat caudal to the pronephros.¹⁰

The limb bud on its first appearance is a nodule about one and a half somites in diameter, and is almost radially symmetrical. It soon acquires greater convexity on its dorso-posterior border (fig. 4), and may be said to 'point' in that direction, though the surface is rounded all over. From this period on growth is rapid. The direction of 'pointing' becomes more marked and the tip of the bud frees itself from the body wall, the axis of the limb making an angle of 30° – 35° with that of the body when viewed from above, and pointing dorsally at about the same angle to the horizontal. The bud elongates into cylindrical form, being attached to the body wall obliquely at its base. During this process the axis of the limb is bent more nearly parallel to the median plane (figs. 5 and 6). As the bending proceeds the distal part of the limb becomes flattened in a plane about 45° to vertical, the dorsal border being nearest the body. A little later the first trace of the digitations appears at the extreme tip of the limb, which becomes more squarish and then slightly concave, the depression representing the notch between the first two digits (fig. 7). The digits elongate rapidly, as does the whole limb, but the joints are at this time not very distinct. While the notch between the fingers is still shallow, the dorsal border of the limb becomes distinctly convex, and at the same time the hand is so twisted as to lie in a vertical instead of an oblique plane. The latter change is in reality partial pronation. The more lateral, which is morphologically the preaxial (radial) border, becomes ventral, the pollex lying on this side. The hand broadens out and the fore arm becomes somewhat flattened also. The elbow joint is now slightly flexed towards the ventral side (fig. 8). Up to this point the limb has no motility, the changes which have been described being due to growth and not to muscular action.

¹⁰ The description of the normal development is given here merely for convenience, and only those phases which concern the experiments are considered. The subject is fully treated in papers by Götte ('79), Strasser ('79), Rabl ('01), with especial emphasis on external form and skeleton. Miss Byrnes ('98 a) has described the relation of the limb bud to the body wall and myotomes in the early stages.

The third and fourth digits appear successively on the ulnar (dorsal) border of the hand, first as nodules which slowly elon-



Figs. 4 to 9 Normal embryos of *A. punctatum*, showing form and posture of the developing fore limb. Description in text. $\times 10$.

gate, the fourth being considerably behind the third in its development (fig. 9). Viewed from above, the arm at this time is

seen to be bowed toward the body. Further changes in the form of the limb are concerned largely with the lengthening of the various segments, notably the digits, and the more distinct demarcation of the arm, fore arm, and manus.

Rotation takes place at the shoulder, the arm pointing more laterally and ventrally, so that the tip of the first digit rests on the bottom. Further rotation at this joint, coupled with flexion at the elbow, brings the manus much further forward beneath the gills, and the animal now rests upon two digits of each limb. The balancers, which serve to support the larva on its belly, are not lost till this stage is reached. The first muscular movements take place at the shoulder before this period, and later, movement at the elbow and wrist joints begins; the limb is then used in crawling, the positions just described being those of normal rest. These changes are completed just about the time the yolk is entirely gone and the larva begins to feed.

SIMPLE EXTIRPATION OF THE LIMB BUD

Mode of operation

The first experiment which will be considered consisted in the simple extirpation of the body wall of the limb region. To perform this operation the scissors are inserted through the outer layers of the embryo at the anterior (cephalic) border of the region to be removed and then the embryo is turned while a circular incision of the desired size is cut. The part of the body wall thus isolated may be readily lifted from the underlying tissue and removed in entirety. Care has to be taken at the upper border of the wound to disengage the limb rudiment from the pronephros, if the latter is to be saved intact. When the limb disc is lifted, both somatopleure and splanchnopleure come away with the overlying ectoderm, since the mesoderm has not split at that time. The operations were mostly done in the stage shown in figure 2, though the age of the embryos used varied from the stage shown in figure 1 to that in figure 3. No difference in the results, which may be ascribed to the difference in age within these limits, has as yet been noted. In the younger

specimens the layers of the embryo are more readily separated, though in the older ones the tissues are of a firmer consistency and can be handled more satisfactorily.

After the disc is excised the wound at first gapes, but a few minutes later it contracts. Then in the course of the next twenty-four or forty-eight hours the ectoderm stretches itself over the wound bed and covers it entirely. There is much variation in the time required to complete this process, and in some cases the wound has been found still partly open four days after the operation. Sections show that the wound is first covered by ectoderm and that the mesoderm creeps in soon after between that layer and the yolk.

The first problem is to determine whether by an operation of this kind the development of the limb can be prevented, and, if so, how the size of the wound affects the outcome of the experiment. Two kinds of operations were done. In the first the limb disc was simply lifted and the wound left without further treatment. In the second the wound bed was afterwards carefully cleaned of all mesoderm cells. In some cases of each kind the pronephros was left intact and in others it was removed. Extirpation of this organ facilitates the cleaning of the wound, but since many cases of non-regeneration with intact pronephros occurred as well as some cases of regeneration without its presence, its influence, if any, upon the development of the limb must be unessential.

Relation of regeneration to size of wound and completeness of removal of mesoderm

The smallest wounds were three somites in diameter, encompassing in all but two cases the region ventral to myotomes 3, 4, and 5. In the two exceptional cases the wound, which was not cleaned, included the area below somites 4, 5, and 6; one of these regenerated and one died. The largest were $4\frac{1}{2}$ somites in diameter, extending from the boundary between the second and third to the vertical line dividing the seventh myotome in half.

TABLE 1

*Showing results of extirpation of circular areas of body wall of given diameter;
wound not covered*

| DIAMETER OF EXTIRPATED AREA | WOUND NOT CLEANED | | | | | WOUND CLEANED | | | | |
|--------------------------------|------------------------|----------------------------|------------------------|-----------|----------------------------|------------------------|----------------------------|------------------------|---------|---------------------------|
| | Cases regener- ated | Cases not re- generated | Dead or dis- carded | Total | Per cent re- generated* | Cases regener- ated | Cases not re- generated | Dead or dis- carded | Total | Per cent re- generated |
| Not recorded... | 11 | 10 | 4 | 25 (21) | 52.4 | 0 | 0 | 1 | 1 (0) | |
| 3 somites..... | 19 | 1 | 4 | 24 (20) | 95.0 | 13 | 12 | 9 | 34 (25) | 52.0 |
| 3½ somites..... | 1 | 0 | 0 | 1 (1) | | 0 | 0 | 0 | 1 (1) | |
| 3½ somites..... | 111 | 23 | 81 | 215 (134) | 82.8 | 3 | 18 | 11 | 32 (21) | 14.3 |
| 4 somites..... | 14 | 2 | 2 | 18 (16) | 87.5 | 2 | 3 | 13 | 18 (5) | |
| 4½ somites..... | 2 | 0 | 0 | 2 (2) | | | | | | |
| Total..... | 158 | 36 | 91 | 285 (194) | 81.4 | 19 | 33 | 34 | 86 (52) | 36.5 |

* In calculating the percentages the number surviving and not the whole numbers of operations has been used. Percentages are given only in those classes where the number of cases is sufficiently large to be of significance.

The standard wound, which was made in the largest number of cases, was 3½ somites in diameter, including the area ventral to the third, fourth, fifth, and half of the sixth myotomes. The wounds of four segments in diameter took in usually the region extending from the third to the sixth somites, inclusive, though in one case the place of the wound was shifted one segment, and in ten other cases a half segment toward the head, thus including in the latter that portion of the body wall between the posterior half of the second somite and the anterior half of the sixth inclusive.

As shown in table 1, the result of the experiment depends in a certain measure both upon the size of the extirpated area and the completeness of the removal of tissue. Taking all of the experiments without reference to size, 81.4 per cent of the cases with ordinary wound regenerated limbs while only 36.5 with cleaned wounds did so. In both sets of experiments, increase in the size of the wound over three somites considerably reduces the proportion of regenerated limbs. High mortality of the large clean-wound class has considerably diminished the number of

cases of this kind available. In these cases the healing is usually very slow. The yolk, being long exposed, begins to disintegrate after a time, and the embryo rarely recovers if this process sets in. It is possible that when a few mesoderm cells are left in, they cover the yolk and facilitate the overgrowth of the ectoderm.

Although the regenerative capacity is dependent to some extent on the size of the wound, there are experiments with wounds of each of the several sizes in which no regeneration took place. Within the limits of these operations it is therefore impossible to say that wounds beyond a certain size preclude regeneration altogether. This can only be said of those cases in which the wound is covered with indifferent skin, as described in another section (p. 432).

It is reasonably certain from a study of the normal development that the cells which ordinarily give rise to the limb bud take origin in the region below the fourth and the neighboring parts of the third and fifth somites. In this region, as the limb bud becomes more prominent, numerous mitoses are found, while the rest of the somatopleure is almost devoid of dividing cells. In those cases in which the region corresponding to the whole of these three somites, or even more, is removed the cells which give rise to the regenerated appendage are, therefore, probably such as in normal development do not participate in its development. Miss Byrnes ('98) has emphasized this in her paper on the hind limbs of *Rana*.

Relation of regeneration to age of embryo at time of operation

There seems to be no definite correlation between the age of the embryo at the time of operation and the occurrence of regeneration. The tabulation here given (Table 2 a), which includes both covered and uncovered wounds shows that when cases are recorded in significant numbers, both positive and negative results are obtained after operation in both older and younger stages.

The number of cases operated in the extreme stages is small and no statistical value can be placed upon the figures there.

TABLE 2A

Showing frequency of regeneration after operations in the several stages

| STAGE | WOUND NOT CLEANED | | | WOUND CLEANED | | | ALL EXPERIMENTS COMBINED | | |
|----------------|-------------------|-----------------------|----------------------|-------------------|-----------------------|----------------------|--------------------------|-------------|----------------------|
| | Cases regenerated | Cases not regenerated | Per cent regenerated | Cases regenerated | Cases not regenerated | Per cent regenerated | Dead or discarded | Total cases | Per cent regenerated |
| 25 | 4 | 0 | | 0 | 1 | | 2 | 7 (5) | |
| 26 | 3 | 0 | | 2 | 3 | | 0 | 8 (8) | |
| 27 | 8 | 2 | 80.0 | 3 | 11 | 21.4 | 4 | 28 (24) | 45.8 |
| 28 | 20 | 0 | 100.0 | 2 | 11 | 15.4 | 20 | 53 (33) | 66.7 |
| 29 | 65 | 12 | 84.4 | 10 | 25 | 28.6 | 41 | 153 (112) | 66.1 |
| 30 | 26 | 5 | 83.9 | 5 | 9 | 35.7 | 28 | 73 (45) | 68.9 |
| 31 | 12 | 3 | 80.0 | 2 | 3 | | 28 | 48 (20) | 70.0 |
| 32 | 4 | 4 | | 2 | 0 | | 4 | 14 (10) | 60.0 |
| 33 | 14 | 10 | 58.3 | 1 | 0 | | 4 | 29 (25) | 60.0 |
| Miscellaneous* | 4 | 5 | | 0 | 2 | | 6 | 17 (11) | |
| Total..... | 160 | 41 | | 27 | 65 | | 137 | 430 (293) | |

* Stage not recorded or else outside above limits.

TABLE 2B

Showing frequency of regeneration after operation in the younger and older groups of stages, respectively

| STAGE | WOUND NOT CLEANED | | | WOUND CLEANED | | | ALL EXPERIMENTS COMBINED | | |
|----------------------|-------------------|-----------------------|----------------------|-------------------|-----------------------|----------------------|--------------------------|-----------------------|----------------------|
| | Cases regenerated | Cases not regenerated | Per cent regenerated | Cases regenerated | Cases not regenerated | Per cent regenerated | Cases regenerated | Cases not regenerated | Per cent regenerated |
| Stage 29 or under... | 100 | 14 | 87.7 | 17 | 51 | 25.0 | 117 | 65 | 54.3 |
| Over stage 29..... | 56 | 22 | 71.8 | 10 | 12 | 45.5 | 66 | 34 | 66.0 |

One point stands out, however, which may possibly be of significance, though no altogether satisfactory explanation is apparent. In the group with wounds not cleaned a higher percentage of regenerating cases is found among those operated in the younger stages, while in the group with cleaned wounds the higher percentage is at the opposite end of the series. This is brought out more clearly when the experiments are divided into but two classes, comprising respectively the cases operated when older than the standard operating stage and those in or below it (Table 2 b). The lower percentage of regeneration in

TABLE 3

Showing time factor for regeneration in the different classes of experiments

| CHARACTER OF OPERATION | NUMBER OF CASES | AVERAGE TIME AFTER OPERATION REGENERATION FIRST OBSERVED | AVERAGE TIME AFTER OPERATION LAST PREVIOUS OBSERVATION | NUMBER OF CASES REGENERATION FIRST OBSERVED AT 10 DAYS OR LATER | NUMBER OF CASES REGENERATION FIRST OBSERVED AT 15 DAYS OR LATER | OLDEST STAGE NEGATIVE OBSERVATION FOLLOWED BY REGENERATION |
|------------------------|-----------------|--|--|---|---|--|
| | | <i>days</i> | <i>days</i> | | | <i>days</i> |
| Wound not covered: | | | | | | |
| Not cleaned..... | 158 | 6.8 | 2.8 | 21 | 4 | 10 |
| Cleaned..... | 19 | 9.2 | 5.4 | 7 | 2 | 15 |
| Wound covered: | | | | | | |
| Not cleaned..... | 2 | 13.5* | 2.5* | 1 | 1 | 4 |
| Cleaned..... | 8 | 9.4 | 6.0 | 3 | 1 | 11 |

* The figures in this class have no significance since in one of the two cases no observation was recorded between the fourth and the twenty-third day.

the older embryos in the not-cleaned series may be due to the fact that the limb bud can be lifted out more cleanly than in the younger stages, so that after-cleaning is less needed. The higher percentage of regeneration after operations in the older stages in the cases with cleaned wounds remains, however, anomalous.

Delay in development after extirpation

Removal of the limb rudiment, if it does not arrest development entirely, naturally retards it to a considerable degree, the delay being on the average greater in the case of the cleaned wounds than in the others (Table 3). In a few cases outward signs of regeneration were noted as early as four days after operation, though usually not until later. Since it was not practicable to observe the embryos each day, it cannot be stated exactly when regeneration did begin in each case. One hundred and fifty-eight cases with ordinary wounds show that the regenerating limb was first noted on the average 6.8 days after the operation, the last previous observation having been made on the average at 2.8 days. In the case of the cleaned wounds, the first observation of regeneration averaged 9.2 days after operation, the last previous observation having been at 5.4

days. This is an average of nineteen cases. Daily observation would undoubtedly have revealed a greater difference between the two classes of cases in time of appearance of the regenerating limb bud. In a total of 177 cases there are but twenty-eight in which regeneration was first noted as late as ten days after the operation, and but six when the period was fifteen days or over. In only four of the twenty-eight cases was the last previous observation made as late as ten days after the operation, and only once was a negative observation, with subsequent regeneration, recorded as late as fifteen days. It is safe to conclude, then, that when regeneration has not become visible from the outside two weeks after the operation it will not occur at all. Nearly all of the cases have been kept alive at least three weeks, many for four, and some as long as twelve (fig. 14), and no instances of regeneration in the later periods of development have been observed.

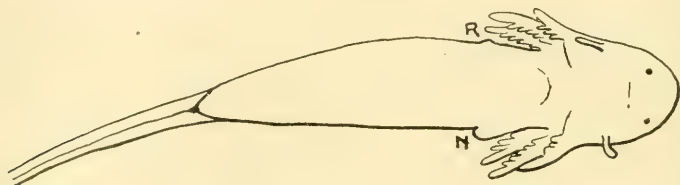
The experiments described in this section show that it is not possible to prevent the regeneration of the fore limb with certainty when the wound is left uncovered, even if the circular area extirpated has a diameter of 4 somites. It may be said in general, however, that the larger the area removed and the more carefully the wound is cleaned of mesoderm the less likely will regeneration occur. This can only mean that the mesoderm cells of the region surrounding the limb bud have, in a gradually diminishing degree as the distance from the limb increases, the potency to form a limb. Their prospective potency is, therefore, greater than their prospective significance. Regeneration, when it takes place, is usually perfect, though subject to delay in a varying degree.

The process of regeneration

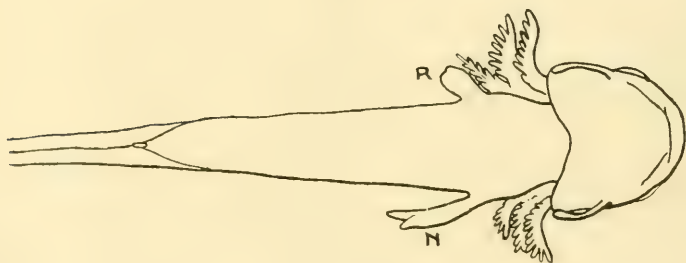
The actual process of regeneration has not been followed in detail, though some observations may be recorded here.

The earliest case (R. E. 127-) which need be considered was preserved six days after extirpation of the limb rudiment. The regenerating limb appears on the surface (fig. 10) as a

small nodule ventral and posterior to the pronephros. In sections it is seen to consist of closely packed cells which have approximately the same yolk content as those of the normal limb on the opposite side and which show numerous mitoses. This indicates that after the defect is covered up by inwandering of peripheral cells (p. 422), the process of regeneration, like the original development of the limb, is dependent upon multiplica-



10



11

Figs. 10 and 11 Embryos with regenerating limb buds, ventral view. $\times 10$. *R*, regenerating limb; *N*, normal limb. Figure 10. Experiment R. E. 127-, six days after operation. Figure 11. Experiment R. E. 129-, fifteen days after operation.

tion of cells in situ rather than upon the continued crowding together of cells from the surrounding regions. The ectodermal covering of the new limb bud, as well as that of the original one, is considerably thicker than the ectoderm of the neighboring region of the trunk.

A case (R. E. 115-) preserved at nine days shows similar conditions, though further advanced. Nerve fibers from two spinal nerves may be traced to the base of the regenerating limb.

A larva killed fifteen days after operation (R. E. 129—, fig. 11) shows traces of digits and the segregation of some of the skeletal elements, though no marked differentiation of tissues. Mitoses are still very numerous and the blood vessels within the limb are unusually large. It is odd that there is as yet no trace of the shoulder girdle, which is already well developed on the normal side. In normal limbs of the size of the regenerating one in this specimen, the girdle can be readily made out.

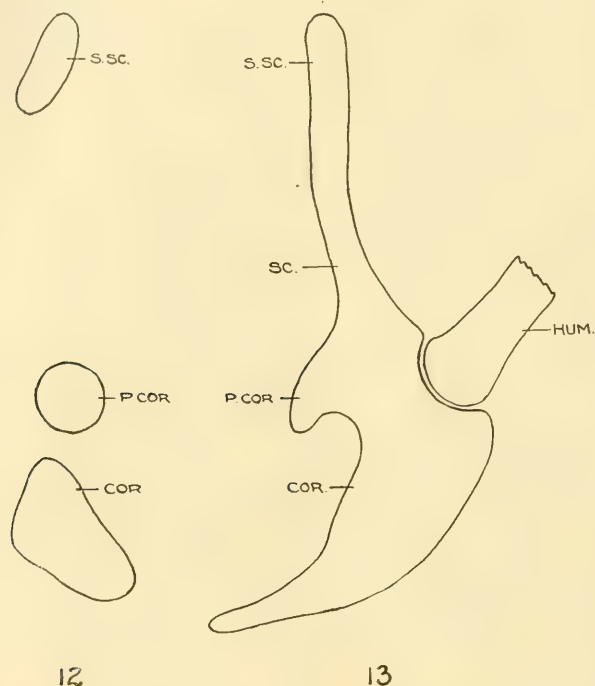
The shoulder girdle in the absence of the limb

In cases where the free limb does not regenerate there is a partial development of the shoulder girdle. This is shown in every case that has been examined, confirming Braus's observations on the Anura.

Some of these cases have been studied in serial sections and others in total preparations of the shoulder region (figs. 12 and 13). The cartilaginous elements found are *a*) the coracoid (*COR*), which has the same general shape and position as the normal, but is not so large; *b*) a small nodule, dorsal to the coracoid at the point where the humerus would normally articulate, representing the procoracoid (*P. COR*), and *c*) a rod-shaped element, external to the pronephros, which is entirely separate from the rest of the girdle and which may be identified with the suprascapula (*S. SC*). The scapula is absent. The ventral trunk musculature is approximately normal, though thinner in the girdle region, and usually it is partly interrupted. A distinct band of muscle running from the ventral end of the suprascapula dorsally and anteriorly just under the epidermis is to be identified with the *m. trapezius*, though in the normal girdle this muscle is attached further ventrally. The conditions found change with the age of the specimen.

In a case preserved eighteen days after extirpation of the limb bud (E. E. 61) there are only two small nodules of cartilage, or rather precartilage, present. One is at or slightly ventral to the level of the normal shoulder joint and represents the coracoid, and the other is between the muscle plates and skin at the level of the notochord, opposite the dorsal extremity

of the normal suprascapula, which it undoubtedly represents in rudimentary form. The pronephros had been removed in this case so that the relation to this organ could not be exactly determined.



Figs. 12 and 13 Outline of shoulder girdle cartilages of an embryo (Experiment E. E. 59), from which the fore limb bud had been removed on the right side and had not regenerated. Specimen preserved twenty-seven days after operation. From a total preparation of the shoulder region. $\times 60$. The cartilages are represented in lateral view projected upon the sagittal plane. *COR.*, coracoid; *HUM.*, humerus; *P.COR.*, procoracoid; *SC.*, scapula; *S.SC.*, suprascapula. Figure 12. Operated right side. Figure 13. Normal left side.

Another specimen (E. E. 26), preserved twenty-four days after operation, shows both the dorsal and ventral elements much better developed. The suprascapula is a long rod of cartilage extending from near the level of the *n. lateralis* ventrally almost to the level of the glenoid cavity of the unoperated side. The coracoid is more massive than in the previous case. The

procoracoid is also present, being represented by a small nodule of cartilage anterior to the ventral end of the suprascapula. The three cartilages are distinct from one another, but are connected by fascia or ligaments.

A case (E. E. 52) twenty-eight days old has a still heavier girdle, especially the ventral portion, which is almost as large as the normal. The procoracoid is fused with the coracoid, and these two ventral elements are not separated very widely from the suprascapula, although the latter cartilage extends hardly so far ventrally as in E. E. 26.

An individual which lived thirty-three days from the time of operation (E. E. 71) shows the suprascapula very well developed, reaching ventrally nearly to the level of the shoulder

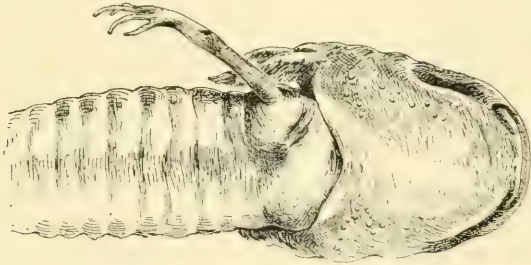


Fig. 14 *Amblystoma* larva (Experiment Tr. E. 53) from which the left limb bud had been removed and not regenerated; preserved eighty-seven days after operation; ventral view. $\times 5$.

joint. However, the ventral part of the girdle is relatively poorly developed in this case, the coracoid and procoracoid being small and still separated from one another.

In a case preserved thirty-nine days after operation (Tr. E. 62-) the dorsal portion of the girdle is well developed and at its ventral end enters into cartilaginous union with the posterior end of the procoracoid. The latter approaches close to the coracoid cartilage and is united to it by a strong ligamentous attachment, though there is no continuity of cartilaginous substance. This is in contrast to E. E. 52, where the coracoid and procoracoid are fused and the suprascapula separate.

The oldest case examined (Tr. Ext. 61-) was killed eighty-

five days after operation (fig. 14, taken from another specimen of like age). In this the shoulder elements are united into a single cartilage, extending from the level of the dorsal surface of the medullary cord to the ventral mid line. On the un-operated side the cartilage is considerably stouter.

Taken together with the results of Detwiler's studies on the development of the shoulder girdle after extirpation of various portions of the limb region, these cases indicate that the operation used in extirpating the limb rudiment leaves in the embryo the rudiments of the suprascapula, the coracoid and the procoracoid. These differentiate into small cartilages, which by a process of hyperplasia gradually extend across the gap intervening between them until they become united into a girdle which is topographically complete.

THE EFFECT OF COVERING THE WOUND WITH SKIN FROM ANOTHER REGION OF THE BODY

In order to block the stimulus to regeneration, presumed to arise from the presence of a defect, the wound made in extirpating the limb rudiment was in a number of cases covered with indifferent skin from the body of another embryo. The covering was taken either from the flank, tailward from the anterior limb region, or from the belly, and consisted of ectoderm with at least a few mesoderm cells attached. In transplanting embryonic skin it is necessary to work quickly. The major operation must be done first and the embryo placed in position ready to receive the graft. Then the skin is excised from another specimen, and transferred directly to the wound in the first embryo, where it must be pressed into place immediately by a silver or glass bar. If this is not done quickly the skin rolls itself into a ball and cannot be used.

Such experiments were made mostly with cleaned wounds of various sizes. The results are given in Table 4, which should be compared line for line with Table 1. From the comparison of the two tables it is seen that covering the wound has a strong tendency to inhibit regeneration. For cleaned wounds of 3 somites in diameter the percentage of regenerating cases is reduced from 52 to 33. When the wound is $3\frac{1}{2}$ somites or more

TABLE 4

Showing results of extirpation of circular areas of body wall of given diameter; wound covered with grafted skin

| DIAMETER OF EXTIRPATED AREA | WOUND NOT CLEANED | | | | | WOUND CLEANED | | | | |
|--------------------------------|------------------------|----------------------------|------------------------|-------|---------------------------|------------------------|----------------------------|------------------------|---------|---------------------------|
| | Cases regener- ated | Cases not re- generated | Dead or dis- carded | Total | Per cent re- generated | Cases regener- ated | Cases not re- generated | Dead or dis- carded | Total | Per cent re- generated |
| Not recorded..... | 2 | 5 | 3* | 10 | | | | | | |
| 3 somites..... | | | | | | 7 | 14 | 3 | 24 (21) | 33.3 |
| 3½ somites..... | | | | | | 1 | 0 | 0 | 1 (1) | |
| 3½ somites..... | | | | | | 0 | 15 | 4 | 19 (15) | 00.0 |
| 4 somites..... | | | | | | 0 | 3 | 2 | 5 (3) | |
| Total..... | 2 | 5 | 3 | 10 | | 8 | 32 | 9 | 49 (40) | 20.0 |

* These three cases were classed as negative in the tabulation previously published (Harrison, '15). They are excluded here because they were not kept under observation for a sufficient length of time.

in diameter, regeneration is altogether blocked, eighteen cases all having given a negative result. To these might be added the five cases given in Table 8, which differ from the present experiments only as to the region from which the covering ectoderm is taken (p. 448).

When the skin is grafted to the wound it soon sticks to the underlying tissue just as a transplanted limb bud does, and often in the course of several hours the wound becomes completely healed. The wandering of the ectoderm over the denuded area, which takes place in the uncovered wounds, is blocked, and probably also the movement of the mesoderm cells. Prevention of the surrounding cells from reaching the proper position by the substitution of other tissues which do not have the potency to produce a limb thus effectually prevents the regeneration of the appendage. The cells immediately surrounding the limb-producing area are evidently unable to form a limb unless they can reach the proper position. On the other hand, indifferent cells in this position cannot produce a limb. It is possible that when no mesoderm is grafted with the skin, the cells of this layer wandering in from the host may in

some cases give rise to a limb. Some of the individual discrepancies in this series of experiments may be due to unconscious varying of this factor.

In experiments in which another limb bud is transplanted into the place of an extirpated normal bud, the grafted tissue must act like a piece of body wall from an indifferent origin in so far as its effect upon the movement of the cells of the host is concerned. It must prevent these cells from wandering into the proper position to form a limb, and hence when transplantations of the limb bud are undertaken with proper precautions as to size of wound and thorough cleaning of the mesoderm, it is safe to assume that the limb that does develop arises from the transplanted material and not from the tissues of the host. The exact determination of the size and character of the wound necessary to prevent regeneration is therefore important for the proper interpretation of any experiments in which the normal limb bud is replaced by a grafted one.

EFFECT OF REMOVAL OF PORTIONS OF THE LIMB BUD

It was scarcely to be expected that an organ having such marked regenerative capacity as the limb rudiment would show any distinct localized effect of the removal of definite portions. A number of experiments have nevertheless been made to test the prospective potency of its parts. The procedure was as follows: The limb area was first bisected by a vertical or a horizontal incision and half of the disc—anterior or posterior, dorsal or ventral—was removed. Some of the wounds were left without further treatment; in others the mesoderm was carefully cleaned off and the wound left to heal; and in still others the denuded area was covered with ectoderm from the flank of another embryo just as in the experiments with whole limb buds. In a smaller number of cases, a small circular area, 1 or $1\frac{1}{2}$ somites in diameter, was removed from the center of the limb rudiment. The first experiments, which were referred to in the preliminary paper (Harrison, '15), were few in number and were made without special cleaning of the wound. They resulted in the development of normal or only slightly defective

TABLE 5

Summary of results of removal of portions of the limb bud. Wounds cleaned

| PART REMOVED | RESULTING LIMB | | | | | | |
|----------------|----------------|--------------|-----------|----------|------|---------|-----------------|
| | Normal | Reduplicated | Defective | Abortive | Dead | Total | Per cent normal |
| Anterior..... | 3 | 6 | 0 | 1 | 4 | 14 (10) | 30.0 |
| Posterior..... | 2 | 1 | 3 | 4 | 3 | 13 (10) | 20.0 |
| Dorsal..... | 1 | 0 | 1 | 10 | 2 | 14 (12) | 8.3 |
| Ventral..... | 8 | 0 | 0 | 2 | 4 | 14 (10) | 80.0 |
| Central..... | 4 | 1 | 1 | 0 | 1 | 7 (6) | 66.7 |
| Total..... | 18 | 8 | 5 | 17 | 14 | 62 (48) | 37.5 |

TABLE 6

Summary of results of removal of portions of the limb bud. Wounds not cleaned

| PART REMOVED | RESULTING LIMB | | | | | | |
|----------------|----------------|--------------|-----------|----------|------|---------|-----------------|
| | Normal | Reduplicated | Defective | Abortive | Dead | Total | Per cent normal |
| Anterior..... | 12 | 0 | 1 | 0 | 4 | 17 (13) | 92.3 |
| Posterior..... | 6 | 0 | 4 | 1 | 4 | 15 (11) | 54.5 |
| Dorsal..... | 5 | 0 | 1 | 4 | 1 | 11 (10) | 50.0 |
| Ventral..... | 7 | 0 | 1 | 0 | 2 | 10 (8) | 87.5 |
| Central..... | 1 | 0 | 1 | 0 | 0 | 2 (2) | 50.0 |
| Total..... | 31 | 0 | 8 | 5 | 11 | 55 (44) | 70.5 |

limbs, but cannot be regarded as a fair sample of what the operation may effect.

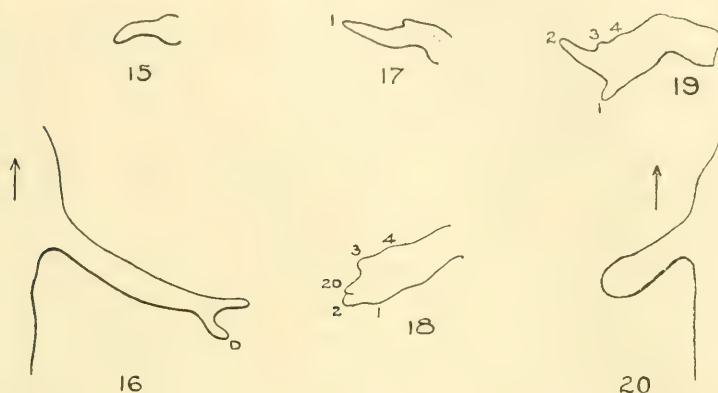
During the season of 1917 the experiments have been greatly augmented in number, and with results which seem rather different. There has been a large proportion of cases with defective or completely inhibited development of the operated limbs, although no specific correlation between the part removed and the character of the defect has been observed. As in the experiments with whole limb buds, a much higher percentage of perfect limbs has resulted when the wound is not cleaned. The results of the experiments in the two classes of cases are given in Tables 5 and 6.

Those with cleaned wounds (Table 5), including all which were covered with grafted skin, will be considered first. In this

group it is obvious that the proportion of normal limbs varies very greatly among the different operations. Thus when the ventral half of the limb bud was removed, eight out of ten resulted in perfect limbs; but when the dorsal half was taken out, only one out of twelve gave rise to a normal appendage. The anterior and posterior halves occupy an intermediate position between the two extremes. These discrepancies are largely, if not entirely, due to the difficulty of exactly halving the material that is to form the limb. In bisecting it vertically the myotomes were used as a guide, and in most cases the incision was made below the middle of the fourth somite, leaving one and a half somites in front of the incision and two behind it. This seems to divide the material more nearly in half than when the incision is made a quarter somite further back. In the case of the horizontal incision it is more difficult to divide the rudiment accurately, because there is no sharply defined landmark. The attempt was made to cut a little below the pronephric swelling. The results show that more of the limb material lies above the cut than below. In other words, if the circular area, centering just below the pronephros and extending from the boundary between the second and third somites to half way through the sixth, is bisected vertically and horizontally, more limb-forming material lies dorsal to the line than ventral, and more anterior than posterior. The lines shown in figure 2 designate more nearly the exact quartering of the material.

The fact that normal appendages resulted in some cases after each kind of operation shows that there is no localization of prospective potencies in the operating stage. Examination of the character of the defects that do arise confirms this conclusion. The large proportion of the latter are defects of the whole limb, which remains a nodule or becomes entirely resorbed. They must be ascribed to the general effect of the operation and not to the removal of any specific material. Seventeen cases out of a total of forty-eight operations (not including cases that died) with cleaned wounds resulted in this way. Of the partial defects, listed under the caption 'defective,' all five affected the hand, especially the digits. The most marked case was one in

which the dorsal half of the limb bud was removed (Rem. E. 27). This was a club-shaped appendage less than half length and without hand (fig. 15). The next most marked defect followed removal of the posterior half of the bud (H. R. E. 12-); here the forearm was slender and but one whole digit was developed.



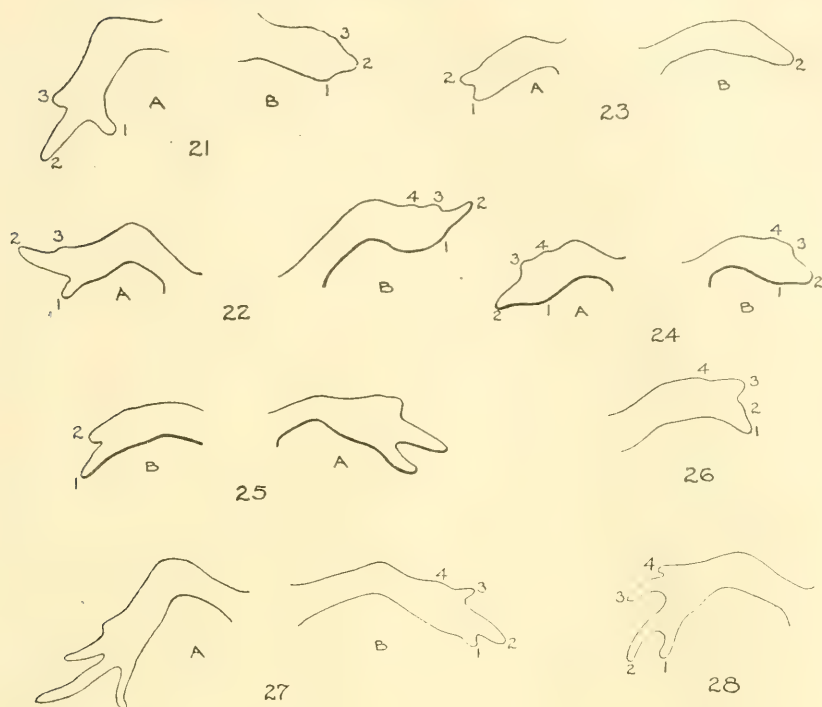
Figs. 15 to 20 Outline views of defective limbs which developed after removal of portions of the limb bud. $\times 17$. 1, 2, 3, 4, ordinal number of the digits; *d.*, reduplicating digit. Figure 15. Experiment Rem. E. 27 (dorsal half removed); lateral view right side, considerably foreshortened; twenty-six days after operation. Figure 16. Experiment H. R. E. 12- (posterior half removed); ventral view; twenty-six days after operation; the arrow points headward. Figure 17. Experiment Rem. E. 13 (posterior half removed); limb amputated and preserved twenty-seven days after operation; ulnar half of hand is very defective, but the limb which regenerated after amputation was normal. Figure 18. Experiment Rem. E. 29 (posterior half removed); lateral view right side, arm much foreshortened; first digit (1) abortive and syndactylous with second, which has a reduplicating branch (2*D*); thirty-one days after operation. Figure 19. Experiment Rem. E. 21 (central portion of limb bud removed); limb amputated and preserved twenty-six days after operation; right limb lateral view. Figure 20. Experiment H. E. 21 - (anterior half removed); right side ventral view, eighteen days after operation; the arrow points headward.

which, however, had a reduplicating branch (fig. 16). In another case of removal of the posterior half (Rem. E. 13) only a single long digit and the stump of a second were developed, the hand being quite defective (fig. 17). Amputation of this incomplete limb was followed, however, by the regeneration of a normally formed one. The two remaining cases showed defects

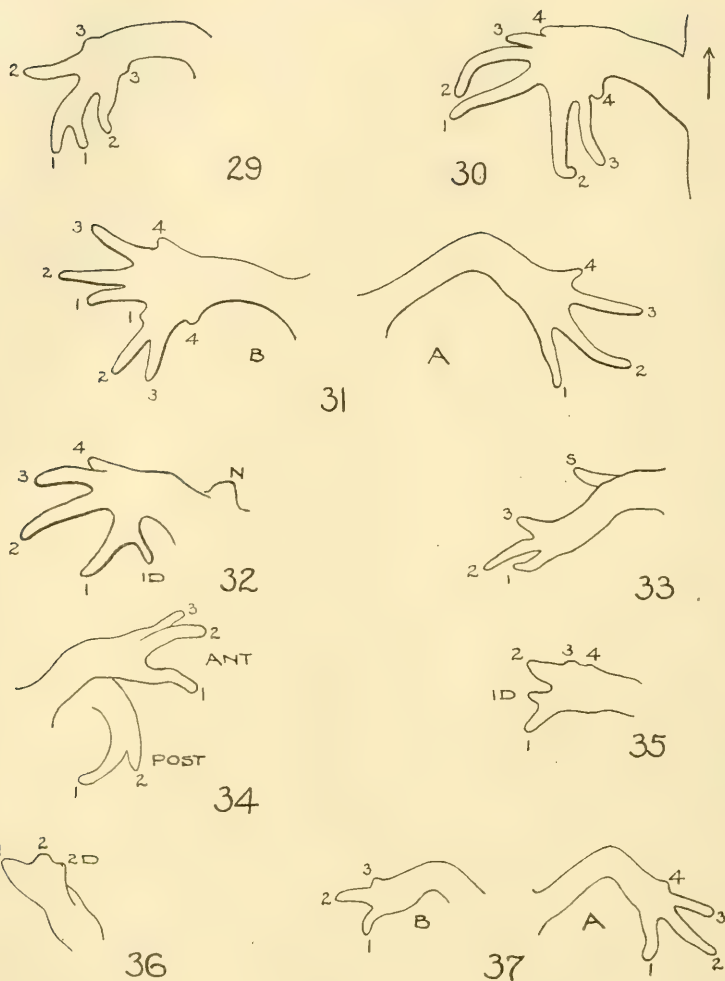
in the first digit. In one (Rem. E. 29), where the posterior half of the bud had been extirpated, the first digit is a mere stump and is syndactylous with the second, which, however, has a small reduplicating branch on the ulnar side (fig. 18); in the other (Rem. E. 21), from which the central portion had been excised, the first digit is short (fig. 19).

The cases with ordinary (not cleaned) wounds (Table 6), like the foregoing, show the highest proportion of defects after removal of the dorsal half of the limb bud. However, the lowest proportion of defectives occurred after removal of the anterior half, a result for which there is no apparent explanation. Removal of the dorsal portion gives a relatively large number of cases of complete suppression of the limb. Of the partial defects, the most marked case (H. E. 21-) has the hand totally lacking, the arm ending as a rounded stump (fig. 20); here the anterior portion had been removed. Another case (H. E. 13-, posterior half removed) appears similar though less clear. In four of the remaining, two of which followed extirpation of the posterior (Rem. E. 2 and 7), one of the ventral (Rem. E. 1) and one of the central (R m. E. 9) portion of the bud, the defect involves only the first digit, which is either absent or short (figs. 21 B-24 B), while the other two cases (H. E. 18-, removal of dorsal half, and H. R. E. 33-, removal of posterior half) have the second digit short (figs. 25 B and 26). One of the cases (Rem. E. 9) shows the same defect on both the operated and the unoperated sides (figs. 24 A and B). In a few of the cases, webbing of the first two fingers occurred (figs. 27 B and 28). This has at times been found in other experiments and even in unoperated limbs, and since the deviation is slight these cases have been classed among the normal.

Turning to the reduplications, we find them concentrated among the cases in which the anterior half of the limb bud had been removed, six out of the eight falling within this group. They include a variety of forms, such as two separate and nearly complete limbs (fig. 34), a single normal limb with a spur attached to the upper arm (fig. 33), and a single limb with merely a branched or double digit (fig. 35). Three of them (Rem. E.



Figs. 21 to 28 Effect of removal of portions of the limb bud (continued). In the double figures *A* represents the limb of the unoperated and *B* that of the operated side. In all cases lateral view. $\times 17$. Figure 21. Experiment Rem. E. 2 (removal of posterior half); upper arm unoperated limb (*A*) much foreshortened; operated limb (*B*) generally less advanced in development, and with abortive first digit (1); seventeen days after operation. Figure 22. Experiment Rem. E. 7 (removal of posterior half); digits of normal limb (*A*) foreshortened in part; first digit of operated limb abortive; twenty-six days after operation. Figure 23. Experiment Rem. E. 1 (removal of ventral half); unoperated (*A*) limb not so long as operated (*B*); which has only one long digit; twenty-nine days after operation. Figure 24. Experiment Rem. E. 9 (central portion removed); both limbs foreshortened; unoperated limb further advanced, but both show defect of first digit; twenty-six days after operation. Figure 25. Experiment H. E. 18 — (dorsal half removed); limb of operated side has short second digit (2); eighteen days after operation. Figure 26. Experiment H. R. E. 33 — (removal of posterior half); second digit (2) is abortive and fused with the first (1); third digit well developed; twenty-one days after operation. Figure 27. Experiment Rem. E. 3 (anterior half removed); operated limb somewhat less developed than normal; syndactyly of digits 1 and 2; twenty-seven days after operation. Figure 28. Experiment H. E. 28 — (removal of posterior half); well-developed limb with syndactylous first and second digits; twenty-eight days after operation.



Figs. 29 to 37 Effect of removal of portions of the limb bud (continued). Lateral view of limbs in all figures except figure 30. $\times 17$. Figure 29. Experiment Rem. E. 48 (removal of anterior half); arm foreshortened, but hand not; radial reduplication of hand; twenty-seven days after operation. Figure 30. Experiment H. E. 6 - (anterior half removed); ventral view; radial reduplication of hand more complete than in last case; thirty-nine days after operation; the arrow points headward. Figure 31. Experiment Rem. E. 52 (removal of anterior half); A, normal tetradactylous limb; B, operated limb, with double hand, considerably foreshortened; twenty-seven days after operation. Figure 32. Experiment Rem. E. 28 (anterior half removed); arm considerably foreshortened, probably reduplicated internally; first digit double (1D); N, nodule at

48, Rem. E. 52, and H. E. 6-) show more or less complete symmetrical reduplication of the hand (figs. 29, 30, and 31). Another (Rem. E. 28) has only the first digit doubled externally (fig. 32), though the unusual thickness of the limb indicates partial internal reduplication. Near the shoulder there is a distinct nodule. The case of the single limb with spur (Rem. E. 17) is interesting in that the spur developed out of a bud which grew posterior to the wound scar and which at first seemed to be the main limb. Anterior to the wound a second bud appeared, first noticed eight days after the operation. On the twelfth day it was still but a nodule, and not till the eighteenth day did it look like a regenerating bud. It finally developed into a normal limb of the proper laterality, the posterior bud remaining attached to it as a spur (fig. 33). The case with two independent limbs (H. R. E. 10-) is fundamentally similar to the foregoing. The main limb bud developed posterior to the wound. Later there appeared anterior to this a second bud. In this case, however, the two buds remained permanently separate. The anterior one gained over the posterior and became a normal limb of proper laterality with good function (fig. 34). The posterior one remained somewhat defective (second digit short and ulnar digits undeveloped), and when last examined alive, thirty-eight days after operation, it showed no movement. The remarkable feature of this case is that both limbs are right-handed, as was probably true of the spur case also, though the spur is too de-

shoulder; thirty-one days after operation. Figure 33. Experiment Rem. E. 17 (anterior half removed); hand foreshortened dorso-ventrally; *S*, spur representing a duplicate limb; forty-three days after operation. Figure 34. Experiment H. R. E. 10 - (removal of anterior half); two left limbs; *ANT.*, anterior member, developed secondarily from anterior border of wound; *POST.*, posterior member developed from the remaining half of the limb bud; forty days after operation. Figure 35. Experiment Rem. E. 18 (posterior half removed); radial digit reduplicated (*D*); limb amputated and preserved twenty-seven days after operation, followed by regeneration of normal limb (fig. 37 *b*). Figure 36. Experiment Rem. E. 16 (central portion removed); reduplicated second digit (*2D*); twenty days after operation. Figure 37. Experiment Rem. E. 18 (posterior half removed); *A*, normal left limb, *B*, normal right limb, regenerated after amputation of abnormal limb shown in figure 35; thirty-three days after amputation.

fective to reveal its laterality. This case differs from the three described above in which the hand is symmetrically reduplicated, one member being a right and one a left, following the rule of mirroring. It is probably of a fundamentally different nature from the others in that the posterior member obviously arose from the remaining half of the limb bud after operation, while the anterior one regenerated from the anterior border of the wound, the two remaining far enough apart not to influence one another. In the case of the true reduplications, the two members presumably arise from a single center which later doubles symmetrically. The other two cases of reduplication are not important. One (Rem. E. 18) involved the first digit only (fig. 35), and the other (Rem. E. 16) the second digit (fig. 36).

In three cases the abnormal appendages which developed were amputated between the shoulder and elbow. One of them (Rem. E. 13, with a very defective hand with only one digit (fig. 17)) and another (R. E. 18 with a reduplicated and a defective digit (fig. 35)) regenerated normal limbs (fig. 37). The other failed to regenerate. These experiments show that such anomalies can hardly be due to the removal of specific organ-forming tissues from the rudiment.

The anomalies are summarized in table 7. From this tabulation it is seen *a*) that defectiveness of the first digit may occur after removal of the posterior or the ventral halves or the central portion of the limb rudiment; *b*) that defectiveness of the whole hand may arise after removal of the anterior, posterior, or dorsal halves; and *c*) that abortive limbs may occur after removal of any of the four halves. As for the reduplications, those of major degree are confined to operations on the anterior half of the limb bud. Minor reduplications, affecting the digits only, occurred in one case after each of three different operations.

It would require a number of experiments many times that included in the above table to give statistical value to the numbers in the several categories, and it is not likely that these can be done in the near future. Possibly the repetition of the experiments on a large scale might show, for instance, a relatively high proportion of defects in digits after removal of the posterior

TABLE 7

Showing distribution of anomalies among the several experiments in removing portions of the limb bud

| CHARACTER OF ANOMALY | PART REMOVED | | | | | | | | | | | | | | |
|--|--------------|-------------|-------|-----------|-------------|-------|---------|-------------|-------|---------|-------------|-------|---------|-------------|-------|
| | Anterior | | | Posterior | | | Dorsal | | | Ventral | | | Central | | |
| | Cleaned | Not cleaned | Total | Cleaned | Not cleaned | Total | Cleaned | Not cleaned | Total | Cleaned | Not cleaned | Total | Cleaned | Not cleaned | Total |
| Syndactyly of first two digits*. | | 2 | 2 | | 2 | 2 | | | | | | | | | |
| First digit defective or absent.... | | | | 1 | 2 | 3 | | | | 1 | 1 | 1 | 1 | 1 | 2 |
| Second digit defective..... | | | | | 1 | 1 | | 1 | 1 | | | | | | |
| Hand defective.... | | | | 1 | | 1 | | | | | | | | | |
| Forearm and hand defective..... | | | | 1 | 1 | 1 | | | | | | | | | |
| High degree of defectiveness—hand absent.... | | 1 | 1 | | | | 1 | | 1 | | | | | | |
| Reduplicated digit..... | 1 | | 1 | 1 | | 1 | | | | | | | 1 | | 1 |
| Reduplicated hand mirrored.. | 3 | | 3 | | | | | | | | | | | | |
| Limb with reduplicating spur... | 1 | | 1 | | | | | | | | | | | | |
| Two distinct limbs not mirrored..... | 1 | | 1 | | | | | | | | | | | | |
| Whole limb abortive or resorbed. | 1 | | 1 | 4 | 1 | 5 | 10 | 4 | 14 | 2 | | 2 | | | |
| Total anomalies... | 7 | 1 | 8* | 8 | 5 | 12* | 11 | 5 | 16 | 2 | 1 | 3 | 2 | 1 | 3 |
| Normal excl. syndactyly..... | 3 | 10 | 13 | 2 | 4 | 7 | 1 | 5 | 6 | 8 | 7 | 15 | 4 | 1 | 5 |
| Per cent normal*..... | 30.0 | 92.3 | 65.2 | 20.0 | 54.5 | 42.9 | 8.3 | 50.0 | 27.3 | 80.0 | 87.5 | 83.3 | 66.7 | 50.0 | 62.5 |

* As in tables 5 and 6 syndactyly is classified with the normal.

half, though the small figures given in the table cannot be deemed significant in this direction. Moreover, this defect, like that of syndactyly, has been found in cases which had not been operated upon at all, and is probably to be regarded as due to slight general disturbances of growth.

On the other hand, the removal of the anterior portion seems to have a definite tendency to bring about reduplication, which is probably due to a more or less complete separation of the remaining portion of the limb rudiment from a regenerative center in front of the wound scar.

Notwithstanding these anomalies, the experiments speak as a whole for the equipotentiality of the four quadrants of the limb bud, at least in a qualitative sense. Quantitatively, the lines which divide the limb-forming tissue equally are anterior, and dorsal to the vertical and horizontal diameters, respectively, of the limb bud as defined by the experiments (fig. 2).

These statements are valid for the free extremity only, and must be held in abeyance with respect to the shoulder girdle, where it is known that localization has taken place in the embryo at the time of operation (p. 429).

While the prospective potency of the limb-forming cells is the same as regards the topographic divisions of the limb, the experiments give no evidence regarding histogenetic potencies. Whether certain cells at the operating stage are already differentiated into cartilage, bone, connective tissue, or muscle-forming elements cannot be determined either by direct observation or by any of the experiments yet devised.

What the prospective significance of the cells of each of the four quadrants of the limb bud is, i.e., what part of the free extremity is formed in normal development out of each, has not yet been determined, though the study of normal embryos points to the view that the distal part of the limb is developed more particularly from cells lying in the posterior half, the ulnar half arising from the dorsal and the radial from the ventral quadrant. It is difficult to devise experiments to test this hypothesis. Grafting of tissue colored by vital stains, such as neutral red and Nile blue sulphate, is not feasible because the stain is all de-

posited in the ectoderm. A few experiments have been made by grafting markers in the form of pieces of notochord, which could readily be recognized, in the mesoderm of the different quadrants of the limb bud, in the hope that they might be located after the limb was developed. As yet these have resulted negatively.

ATTEMPTED SPLITTING OF THE LIMB BUD

Some years ago, in experimenting with larvae of the anuran, *Pelobates*, Tornier ('05) found that by making a deep incision through the hind-limb rudiment and the base of the tail he could produce double appendages.

A few experiments have been made for the same purpose in connection with the present study. The material has necessitated, however, a rather different mode of operation, and the results have proved to be different.

The limb bud was deeply incised through the middle, either dorso-ventrally or antero-posteriorly, and a narrow strip of tissue including both ectoderm and mesoderm was removed. Thirteen experiments were made. Six were lost by accident eleven days after the operation, but all of them had at that time normal limbs on the operated side. The other cases were kept for sixteen or eighteen days and again all had normal limbs, though in four cases development was somewhat retarded. Nine of the embryos were in the oldest stage used (fig. 3) at the time of operation. The wound usually left a distinct scar or groove running across the limb bud, which, however, was obliterated after several days. In no case did the operation result in reduplications. The difference between these results and those of Tornier may be ascribed to the fact that in the case of the latter the operation was more radical and done upon older embryos, so that the chance of the divided limb rudiment healing together completely was much less.

EFFECT OF SUPERIMPOSING LIMB BUDS

In experiments upon early embryonic stages the most usual test of equipotentiality of the parts has been the development

of a whole organism out of any part of sufficient size. Another test, more difficult and less frequently tried, is the rearing of a single organism from two eggs or embryos which have been made to fuse together.¹¹

The experiments described in the foregoing section have shown that any half of the limb bud can give rise to a whole limb. Those to be taken up now demonstrate that two limb buds fused together will develop into a normal single limb.

The operation of superimposition or fusing together of two limb buds is carried out as follows: A circular incision of the proper size ($3\frac{1}{2}$ segments in diameter) is made through the ectoderm of the fore-limb region, care being taken to injure the underlying mesoderm as little as possible. The ectoderm may then be readily stripped from the middle layer by inserting the points of the scissors or needle at the dorsal part of the cut and tearing the ectoderm away. Often a few mesoderm cells, especially from the ventro-posterior quadrant, come off with the ectoderm, but the greater part of the cells composing the limb bud remain in place and not infrequently every cell is left intact. An entire limb bud from another embryo is then grafted in the usual way over the mesoderm of the limb thus exposed, and such grafts heal in very readily. The results of these experiments differ according to the orientation of the grafted bud, in harmony with the rules of laterality (Harrison, '17). At present only the cases in which the grafted bud has its normal orientation will be considered.

Five such experiments were made, all of the embryos surviving and giving the same results. Normal limbs developed which at first showed difference in size, but this difference was after a time obliterated. The greater massiveness of the double bud was usually apparent the day after operation and was most marked about three or four days later. In two cases it is recorded as persisting for twelve days, though the difference from the normal gradually diminishes, disappearing entirely by the time the yolk is entirely gone, i.e., about eighteen days.

¹¹ Cf. Morgan, '95; Driesch, '00, '10; Goldfarb, '14.

It is clear from these experiments, even though small in number, that a single perfectly normal limb will regularly develop out of two limb buds fused together.

EFFECT OF REMOVING THE ECTODERM ALONE

In the early days of the study of the limbs the question whether the first sign of their appearance was in ectoderm or mesoderm was much discussed as one of phylogenetic significance. Whatever our attitude toward such questions may now be, it is important from the standpoint of developmental mechanics to inquire whether the factor which determines the development of the limb is located in the outer or the middle germ layer.¹²

Comparison of the experiments with cleaned and with uncleaned wounds, in which the ectoderm is treated the same way in both series while the mesoderm is differently handled, already suggests what the answer will be. The experiments described in this and the following sections are intended to add further and more direct evidence that the power to produce the limb rests primarily in the mesoderm.

It is not always possible to avoid injuring the mesoderm to a slight extent in the operation of removing the ectoderm. Therefore the experiments in which it was attempted to remove the ectoderm alone differ only in degree from the cases of simple extirpation with non-cleaned wounds. Five operations of this kind were performed, and in all five cases limbs developed promptly, though there was some delay as compared with the normal. The wound was not covered with grafted skin in any of the cases.

¹² Braus ('09) has touched upon this question in connection with his study of the origin of the skleroblasts of the limb. On p. 165 he says, "In einer späteren Arbeit werde ich nachweisen können, dass in älteren Stadien ebenfalls das Ectoderm nicht für das Zustandekommen eines typischen Skelets notwendig ist; denn dasselbe bildet sich eben so gut unter einem ortsfremden Ectoderm, welches experimentell gegen die typische Nachbarepidermis ausgetauscht wird, und in grossen Entfernung von jeglicher Haut wie in der ungestörten Entwicklung." The paper dealing especially with this work (Braus, '08) is unfortunately not accessible to me at present.

EFFECT OF REMOVAL OF MESODERM ALONE

In order to remove the mesoderm alone, the ectoderm covering the limb region is first incised around three-fourths of its circumference. It is carefully lifted from the underlying mesoderm and left hanging by its ventral border. The mesoderm is then removed from the region below the pronephros, all loose cells being cleaned off, as in experiments already described, and then the covering layer is finally stretched back into place and held for a short time by a glass bar. The ectoderm contracts considerably while the wound is being cleaned, but with the aid of a fine needle it can usually be drawn over the wound. In some cases perfect healing was obtained in less than an hour. In others small areas of yolk were found still uncovered on the day after the operation. The quickness of the healing seems, however, to have no effect on the result, for in the three cases in which regeneration occurred, the healing was characterized as good, fair, and bad, respectively, while the cases of non-regeneration followed both good and fair healing.

Twelve experiments were made, in two of which the embryos died. In the ten remaining cases the wounds were of different sizes, varying from three to four segments in diameter, bounded as in the simple extirpations (p. 422). The results are given in table 8.

In five cases the cleaned area was of the smallest size, extending from the line between the second and third somites to that between the fifth and sixth. Three of these gave rise to regenerated limbs, while the other two did not. None of the other cases, which had larger wounds, regenerated.¹³ In six of the cases which showed no regeneration the pronephros was left intact and in only one case was it removed. These experiments differ from those described in the third section (p. 432) only with respect to the region from which the ectoderm covering the wound is taken. The results are in full agreement, and the corresponding figures given in table 4 and table 8 could with propriety be combined.

¹³ In my preliminary note (Harrison, '15), on the fifth line from the bottom of p. 542, the words 'over three' should be substituted for 'four.'

TABLE 8
Showing effect of removal of limb mesoderm

| SIZE OF WOUND | CASES REGENERATED | | | | CASES NOT REGENERATED | | | |
|--------------------------------|-------------------|------|------|-------|-----------------------|------|------|-------|
| | Healing of wound | | | Total | Healing of wound | | | Total |
| | Good | Fair | Poor | | Good | Fair | Poor | |
| 3 somites..... | 1 | 1 | 1 | 3 | 2 | | | 2 |
| 3 $\frac{1}{4}$ * somites..... | | | | 0 | | 1 | | 1 |
| 3 $\frac{1}{2}$ somites..... | | | | 0 | 1 | 1 | | 2 |
| 4 somites..... | | | | 0 | | 2 | | 2 |
| Total..... | 1 | 1 | 1 | 3 | 3 | 4 | | 7 |

* In the table previously published (Harrison, '15, p. 540) in the first column fifth line read 3 $\frac{1}{4}$ instead of 3 $\frac{1}{2}$.

While the number of cases is not large, it is beyond doubt that the presence of the normal ectoderm over the denuded limb region no more incites the development of a limb than does the presence of ectoderm from a distant region.

TRANSPLANTATION OF THE MESODERM

Transplantation of the whole limb bud, ectoderm and mesoderm, results, as is well known, in the development of a limb in the new position. Transplantation of one or the other of the two layers should afford additional evidence, more cogent than that already given, as to the potency of the several layers in determining the development of the appendage. Only one of these experiments, the transplantation of the mesoderm, has been tried; the negative results recorded with reference to the ectoderm in the previous section are thought to be sufficient evidence from that side.

In order to transplant or inoculate the mesoderm into some other region of the body, a pocket is first made under the skin of the embryo by sticking the points of a pair of fine scissors obliquely through the ectoderm and slightly opening them. The position chosen for this pocket was in most cases the flank of the embryo at the lower border of the muscle plates. In four cases, all of which resulted negatively, it was made above the

eye. After the first embryo has been prepared, the ectoderm is removed from the limb region of another specimen, with as little injury as possible to the mesoderm, and the latter is cut out from below and behind the pronephros and transferred as a single piece to the pocket. It is often difficult to get this small mass of tissue inserted, because it is very sticky and is liable to be pulled out when the instrument is withdrawn. Having a small hole in the distal side of the pocket facilitates a deep insertion and consequently the retention of the transplanted cells. Healing of wounds of this character is rapid and without secondary complications. The mesoderm cells are, however, rather loosely held together and a considerable amount of disintegration may occur—more than when the limb bud is transplanted in toto.

The results of the experiments were as follows:

| | cases |
|---|-------|
| Embryo died prematurely..... | 4 |
| Resorption of transplanted tissue..... | 5 |
| Small nodule developed..... | 3 |
| Long appendage without digits..... | 1 |
| Limb of approximately full size with digits, usually showing reduplication..... | 6 |
| Total..... | 19 |

These results are not essentially different from those obtained when the whole limb bud is transplanted, except that the limbs which do develop when the mesoderm is taken alone are more likely to show deformities. This was to have been expected in view of the difficulties of handling the mesoderm without the firm ectodermal covering to hold it together. Since the individual cases are of interest their histories will be presented separately.

Experiment Tr. Mes. 1. May 9, 1912. Mesoderm from left limb bud transplanted to left side. Some oozing from wound three hours afterward, indicating loss of tissue.

May 12. Small lump in region of graft.

May 15. Transplanted limb is getting much longer and is not much below the normal one in size.

May 18. Beginning to show digitations.

May 21. Larva has marked spinal curvature, but seems otherwise

healthy. Transplanted limb has two well-marked digits and beginning of third. Limb not so long or so far advanced as the normal one. Good circulation.

May 25. The specimen was preserved on account of the deformity of its back, which rendered its existence precarious (fig. 38).

Transplanted limb is a left, having preserved its original laterality. It has a distinct third digit and the beginning of fourth, and on the radial side there is a reduplication of digits. The whole hand is, therefore, nearly symmetrical. Transverse sections show a small coracoid and a very shallow glenoid fossa, but the scapular portion of the girdle is not developed. Differentiated muscle fibers are present in the limb, but no nerves have been found.

Experiment Tr. Mes. 4. May 11, 1912. Right limb mesoderm to right side.

May 15. Grafted tissue not very prominent.

May 21. Limb bud 'points' anteriorly.

May 27. Digitations beginning; dorso-ventral doubling.

June 7. Specimen preserved.

The arm as a whole is a left, i.e., its laterality has been reversed (figs. 39 and 40). The radial digit is reduplicated on the radial border. There is another reduplication consisting of a long digit and a nodule, mirrored from an ulno-palmar plane.

In the normal limb on this side the first two digits are syndactylous, the first being short.

Experiment Tr. Mes. 5. May 11, 1912. Mesoderm from right limb bud implanted on right flank.

May 12. Wound still slightly open; transplanted tissue a good hump.

May 21. Transplanted tissue has grown and points more distinctly anteriorly.

May 27. Good circulation; two digits show; limb looks to be of normal form.

June 7. Specimen preserved.

Three well-marked digits are present with trace of fourth. The first two digits are webbed; otherwise the limb is normal (fig. 41). It is clearly a left, its original laterality having been reversed. There was no evidence of motility of the implanted limb before killing.

The preserved specimen was cut into frontal sections, examination of which shows that the shoulder-girdle cartilage is fairly well formed; the ventral (coracoid) portion is more extensive than the scapula, which is only slightly developed. Pronephric tubules, seen near the base of the limb, indicate that part of the pronephros was transplanted with the limb cells. Muscle tissue is well developed in the limb, though no nerve fibers seem to be present.

Experiment Tr. Mes. 16. May 13, 1914. Left limb mesoderm transplanted to right side.

May 14. Perfectly healed; small nodule caused by transplanted tissue.



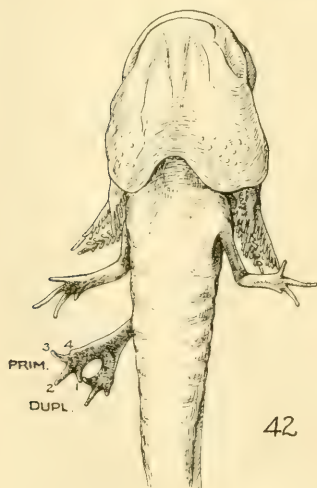
38



39



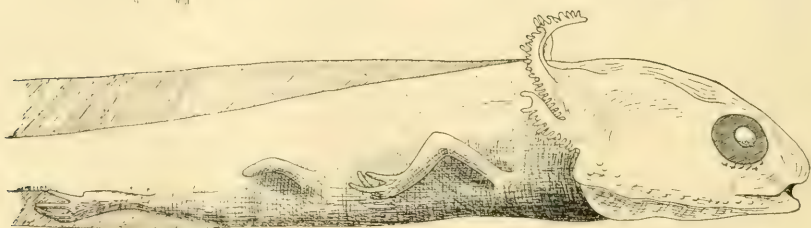
40



42



41



43

May 20. Transplanted tissue growing well, 'points' anteriorly.

May 22. Growth considerable; reduplication beginning near base.

May 25. Limb consists of two almost equal parts branching near base; anterior member is bidigitate.

June 1. A perfect limb with reduplication of forearm and manus. Both members are tridigitate, with indications of fourth digit (fig. 42). The anterior member (*PRIM.*) which is the primary one, is a perfect left (original laterality of the tissue), the other (*DUPL.*) a right.

June 6. Larva preserved.

The specimen was cut into serial sections. The coracoid portion of the shoulder girdle is well developed and the glenoid fossa is marked. Dorsal to the joint there is no cartilage. Well-developed muscles run from the coracoid to the humerus, but the upper arm is almost devoid of muscle fibers. On the other hand, the forearm muscles are well developed. No nerve fibers have been found,

Experiment Tr. Mes. 17. May 19, 1914. Mesoderm of left fore-limb to right side.

May 25. Transplanted bud sticks out almost straight from side of body.

June 1. Limb has grown considerably, but will probably be defective as to digits.

June 6. Unchanged. Limb cut off just below elbow.

June 29. Larva has grown well. Limb healed, but no hand has developed. It shows the elbow bend and has the posture of a normal right limb (fig. 43).

Experiment Tr. Mes. 18. May 19, 1914. Mesoderm of left limb-bud to right side.

May 20. Healing perfect; transplanted tissue a good nodule.

May 22. Nodule increasing markedly.

May 25. Transplanted tissue growing and points toward tail.

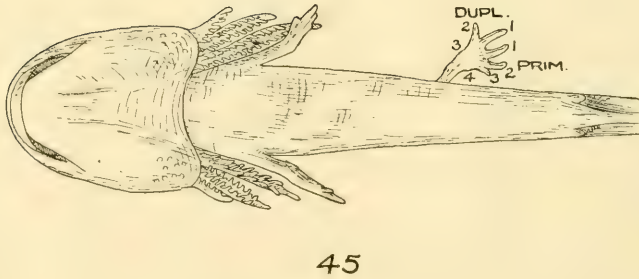
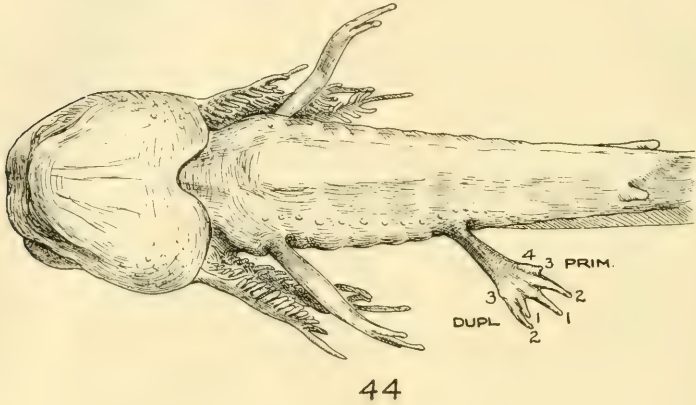
Figs. 38 to 43 Amblystoma larvae showing supernumerary limbs developed from inoculated mesoderm cells of the limb bud. $\times 7\frac{1}{2}$. Figure 38. Experiment Tr. Mes. 1; the supernumerary limb is primarily a left (from left-handed tissue), but the hand is symmetrically reduplicated; sixteen days after operation. Figure 39. Experiment Tr. Mes. 4; supernumerary limb, primarily a left, though the inoculated tissue was from the right side; irregularities in the digits; twenty-seven days after operation. Figure 40. Same experiment, ventral view. Figure 41. Experiment Tr. Mes. 5; the supernumerary limb is a nearly normal left developed on the right side of body from tissue of right side. The only abnormality is the syndactyly of the first two digits; twenty-seven days after operation. Figure 42. Experiment Tr. Mes. 16. Ventral view of larva showing supernumerary limb reduplicated from elbow down; *PRIM.*, primary member, a left (from left handed tissue); *DUPL.*, secondary member, a right; twenty-four days after operation. Figure 43. Experiment Tr. Mes. 17. Supernumerary limb regenerated after amputation, a right (from left-handed tissue) with abortive hand; forty-one days after inoculation, twenty-three days after amputation.

May 27. The limb is bidigitate or bifurcated.

June 1. Limb is short, hand reduplicated and digits irregular.

June 6. Specimen preserved.

The double hand (fig. 44) is approximately symmetrical. The pos-



Figs. 44 and 45 Larvae with supernumerary limbs developed from inoculated mesoderm (continued). $\times 7\frac{1}{2}$. Figure 44. Experiment Tr. Mes. 18. Supernumerary limb with double hand; *PRIM.*, primary member, a right, developed from left-handed mesoderm; *DUPL.*, reduplicating members; eighteen days after operation. Figure 45. Experiment Tr. Mes. 19. Supernumerary limb with double hand; *PRIM.*, primary member a left, developed from right-handed mesoderm; *DUPL.*, reduplicating members; forty days after operation (development very slow).

terior member (*PRIM.*), which, judging by the first direction of pointing, is the primary one, is a right, the original laterality having been reversed. The first two digits are normal, the third is distinct, and the fourth a slight eminence. The other member (*DUPL.*) is not so far developed. The first two digits are webbed and the first consider-

ably shorter than normal. Examination of frontal sections shows that only the coracoid portion of the girdle is developed. Shoulder muscles are present, but no nerves have been found. There are two ulnae, one on each side of the somewhat thickened radius.

Experiment Tr. Mes. 19. March 3, 1915. Mesoderm of right limb to left side.

March 16. Growth of transplanted tissue has been comparatively slow, though in the past three days considerable.

March 19. Limb still short, though showing beginning of digitations.

March 29. Arm very short; three long digits, of which the most ventral and posterior is probably a reduplication. On the dorso-anterior border the third and fourth digits show, the latter but very slightly.

April 12. Specimen preserved.

This is similar to the previous case (Tr. Mes. 18), but the limb is shorter and the elbow bend more distinct. There is an approximately symmetrical radial reduplication of the hand, which is broad and paddle-like. The arm points posteriorly and laterally. The posterior member is probably the primary one and has had its laterality reversed, being a left. The digits are further developed in this hand, both the third and fourth being distinguishable (Fig. 45).

In viewing the above seven cases as a whole, it is seen that, while no one is absolutely normal, they are all sufficiently developed to show their specific form. One alone is badly deficient, two show reduplications which are slight, three others reduplications which are more marked, involving in one case the whole forearm and hand. One case (Tr. Mes. 5) has no reduplication and is normal except that the first two digits are held together by a web throughout their length. This abnormality is, however, not infrequently found in limbs that have not been operated upon.

These results show clearly that the specific limb-forming tissue is the mesoderm of a certain region of the body wall and not the ectoderm. When it is considered that there is much loss of tissue in the operation and that what remains must often be to a great extent mixed up, the most striking thing is that the limbs which develop from such inoculations should be so nearly normal. These experiments thus afford additional evidence for the equipotentiality of the elements constituting the limb bud.

No attempt was made to orient the engrafted tissue, if indeed this were possible, but the results which have been obtained may be interpreted in accordance with the results from transplanting the whole limb bud, where the orientation of the graft is known (Harrison, '17). In three of the cases the limb tissue was grafted on the same side of the embryonic body; one of these yielded a limb of the same side, while in the other two the laterality was reversed. In four cases the tissue was implanted on the opposite side of the embryo; in one of these the limb preserved the original laterality of the tissue, while in the remaining three reversal occurred.

CONCLUSION

The purpose and results of each of the experiments having already been pointed out, it remains only to state briefly their significance as a whole.

The tissue which is destined normally to form the fore limb has been delimited, and within the period of development dealt with in the experiments, it has been shown to be a self-differentiating system. It is a group of mesoderm cells formed as a proliferation of the somatopleure, and no specific stimulus from any particular portion of the ectoderm is necessary for its development. The exact boundary of the embryonic tissue which normally enters into the limb cannot be determined by the present methods; for, surrounding the group of cells which constitutes the limb bud, there is a zone of mesodermal tissue, which, in case of removal of the original limb rudiment, may move in and assume the character of the excised material, giving rise after a time to a normal limb, as was first shown by Miss Byrnes ('98 b) in the case of *Rana* embryos. The limb rudiment may be thus regarded not as a definitely circumscribed area, like a stone in a mosaic, but as a center of differentiation in which the intensity of the process gradually diminishes as the distance from the center increases until it passes away into an indifferent region. Many other systems, such as the nose, ear, hypophysis, gills, seem to have the same indefinite boundaries which may even overlap one another.

Self differentiating as is the system as a whole, the parts within the system do not constitute a developmental mosaic, with the exception of certain portions of the shoulder girdle. The system itself is equipotential, as shown by the two tests to which it can be subjected; a whole will develop out of a part, and a single normal whole will develop out of two separate rudiments when fused together. The experiments of extirpating half buds and of superimposing buds, respectively, make this clear.

The limb rudiment, therefore, is an entity, which, except for its dependence for nourishment, is independent of its surroundings in the attainment of its specific form. In one important respect it has been found to be influenced by its position in the organism as a whole, and that is as regards its relations of symmetry and its power to form reduplications. The study of these phenomena will be the subject of a continuation of the present work.

SUMMARY

The fore limb of *Amblystoma punctatum* develops normally as a thickening of the somatopleure centering in the area ventral to the fourth myotome and extending over into the regions ventral to the third and fifth.

Numerous mitoses indicate that the growth of the limb bud is due to rapid proliferation of the cells in situ rather than to inwandering from surrounding territory.

Extirpation of the tissues of this region results in disturbances of development in the limb, the intensity of which depends upon the size of the wound, the care with which it is cleaned of mesoderm cells, and whether it is covered with grafted ectoderm or not.

If the wound is small or not entirely cleaned of mesoderm, a normal limb usually develops (regenerates) with some delay. After more radical operations the limb regenerates much less frequently.

When nothing further is done beyond excising the circular disc of tissue constituting the limb rudiment, the limb has been found to develop subsequently in 81.4 per cent of the cases

(table 1). When all scattering mesoderm cells are removed, the limb develops in but 36.5 per cent of the cases.

Covering of the wound with ectoderm from the flank further reduces the probability of regeneration. For wounds of 3 somites in diameter the percentage of positive cases is reduced from 52 to 33, and development is entirely prevented by covering wounds of $3\frac{1}{2}$ somites or over.

Even when the free appendage fails to develop, parts of the shoulder girdle are formed.

It is concluded that in these operations the cells that normally give rise to the limb are removed. In the subsequent process of wound healing cells in the surrounding zone move in and ultimately form a new limb bud. Around the limb-forming cells there is thus a zone of tissue which has the power, in gradually diminishing intensity toward the periphery, to form a limb vicariously.

When half of the limb bud is removed, disturbances of development may occur, but in many cases normal development of the limb follows.

These disturbances vary all the way from complete suppression of development to slight retardation. Abnormalities, such as defective digits or hand and reduplications, also occur.

There is no distinct correlation between the part of the limb bud removed and any particular defect.

Reduplications are most frequent after extirpation of the anterior half.

Normal development may occur after excision of any half of the limb, but is more frequent in case of removal of the ventral half.

The limb-forming material is divided into approximately equal parts by lines anterior and dorsal, respectively, to the vertical and horizontal diameters of the limb disc $3\frac{1}{2}$ somites in diameter (fig. 2).

On the whole, these experiments show that, while quantitatively the limb-forming tissue is unequally distributed in the four quadrants of the area usually extirpated, there is no qualitative difference in the potencies of the cells of the four quadrants.

Splitting of the limb bud by a vertical or horizontal incision may cause slight delay in development, but does not produce reduplications.

A single normal limb will develop from two fused limb buds when one is superimposed upon the other in proper orientation.

Removal of the ectoderm covering the limb bud has no effect upon the development of the limb beyond causing slight delay.

Removal of the mesoderm of the limb bud, when complete, prevents the development of the limb even when the proper ectodermal covering is healed back over the wound.

Transplantation (inoculation) of mesoderm from the limb region to a pocket under the skin of the flank results in many cases in the development of limbs. In about half the cases which live this inoculated tissue is resorbed or does not develop further.

The limbs which develop show a large proportion of reduplications; one case was perfectly normal except for syndactyly of the first two digits.

Taken together, the experiments show that while the ectodermal covering is indifferent, the mesoderm of the limb bud constitutes a specific self-differentiating system, which, however, in itself is an equipotential system in Driesch's sense.

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THE INFLUENCE OF EXCESSIVE SEXUAL ACTIVITY OF MALE RABBITS

1. ON THE PROPERTIES OF THE SEMINAL DISCHARGE

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INTRODUCTION

During the past two years the animal breeding laboratory of the department of Animal Husbandry of Iowa State College has carried on work aimed to throw some light on the effects of excessive sexual activity of the male (1st) on the properties of the seminal discharge and also (2d) on the nature of the resulting offspring. This paper is a report of the first-mentioned line of work. A report by one of us on the nature of the offspring appears in the same number of this Journal (Hays, F. A. The influence of excessive sexual activity of male rabbits: II. On the nature of their offspring.) The attributes of the semen and its spermatie content, as influenced by heavy sexual service, which are here reported on are as follows:

The amount of seminal discharge;
The number of sperm¹ per unit volume of semen;
The type of motion and the rate of motion of sperm in fresh semen;

Duration of motion of the sperm;
The certainty of pregnancy induced by the sperm, and
The size of litter from various services.

References to previous published observations will be made under the section on which they most directly bear.

GENERAL PLAN OF EXPERIMENT

Rabbits were selected as the experimental animal: They are large enough to furnish semen sufficient in quantity on which to work, and yet thrive well and reproduce rapidly in close confinement and at slight expense of maintenance. The great majority of the observations were made upon three males. In general the plan was to have the male accomplish, in as rapid succession as possible, a certain number of preliminary 'services' and then to mate him once to the breeding female from which the litter was desired. The 'end services' from which litters were secured for the progeny studies were the 5th, 10th, 15th and 20th, and about an equal number of 1st-service litters were obtained as controls. However, when semen studies were made it was aimed to recover specimens from the 1st and from every 5th service thereafter; thus, in a series of 20 services, five specimens of semen would ideally be recovered for study. This ideal set of specimens from a 20-service series was seldom obtained, however.

In order to provide females for the 'preliminary matings' special provisions were necessary. A single female rabbit will sometimes receive the male as many as fifteen times in the space of about two hours; but, on the other hand, a female may, though she shows every indication of 'heat,' refuse the male after three or four services, and it is not feasible to commence a

¹ Throughout this work the word sperm will be used instead of the technically more correct but cumbersome spermatozoa.

series of fifteen or twenty preliminary matings without having at least two females in heat in addition to the female which is to receive the 'end service. Moreover, inasmuch as the semen specimens were recovered from females after a natural service by the male, it was necessary to have an unused female for every five services made, or at least for every specimen of semen which is to be recovered. In practice a long series of matings was never commenced unless at least three and preferably four or five females were in heat at the same time. The oestral period in rabbits recurs every two weeks if the female does not conceive, and the period of gestation is about thirty-one days. Females will always receive the male the day following parturition, but females in this condition may not be used for recovery of semen because of the presence of large amounts of blood and other fluids residual from the foetal nutrition. Thus, to carry on the work with normal females, an excessively large stock of animals would be necessary. To cut down the number of females necessary to provide the preliminary matings a considerable number were operated upon. The Fallopian tubes were sectioned and the free ends sutured. This operation absolutely prevented conception and for a year or so after the operation the sexual behavior of the female was for the most part otherwise unchanged, and heat recurred with fair regularity about every fifteen days throughout the year. Females treated thus proved to be admirably adapted for our use.

There were some exceptions to the rule in the behavior of the operated stock. A certain agouti female, for example, could be induced to accept the service of the male at almost any time if the male were aggressive. This female apparently went through the regular oestrous cycle, at which time she was much more ardent toward the male and would accept many more copulations than at other periods. A certain sooty female also showed a similar tendency. Attempts to secure copulation with other operated females when not in heat were unsuccessful. After about a year of incessant copulation many of these operated females began to show great pugnacity towards the males, and often, though obviously in heat, refused to accept them alto-

gether. When this behavior developed such females were discarded and replaced by newly operated stock. Post-mortem examinations of some of the discarded females showed no easily apparent changes in their reproductive organs to account for their changed behavior toward the males. Many of them had become very fat and rather sluggish. Altogether about twenty-five females were operated upon. About an equal number of females were used for 'end matings.'

RECOVERY OF SEMEN

There are several methods which have been used by investigators and others for securing specimens of mammalian semen. Ivanoff ('07) recommends highly the vaginal sponge, and advocates the same for use in the horse, the cow, and the sheep. An attempt was made to adapt this method to the rabbit by packing the vagina with absorbent cotton. The method was not successful. The size of the piece of sponge which can be placed deep in the vagina is too small to absorb and yield up sufficient semen on which to work, and if larger pieces are used they remain too near the exterior and prevent penetration by the male. The condom (breeding bag of stallion grooms) has been used much by horse breeders when practicing artificial insemination of females. Good membranes of this sort were prepared from the caeca of chickens, but it was found impossible to use them on the males; neither could they be held in position as a 'false lining' of the vagina at the time of coition. Detlefsen ('14) successfully secured for examination specimens of semen from many different males by making a slight incision in the epididymis and expressing a few drops of its contents. This method will not answer when, as in the present case, a large number of specimens must be taken from a single male. In the case of the rabbit, ejaculation cannot be induced by artificial stimulation.

The method finally adopted and used in all cases was the old and simple one—recovery by catheter. A small-diameter rubber catheter moistened in a saline solution was introduced for a distance of about 16 to 20 cm., thus penetrating as far as the os.

This operation may generally be accomplished, but often folds of the vaginal wall are encountered and much patience is necessary; in very few cases was failure to introduce the catheter deep into the vagina complete. In very rare cases the catheter penetrates the bladder, rather than passing over the papilla, in which event the female in hand is spoiled for recovery of semen that day, because even a small amount of urine remaining in the vagina is highly inimical to the sperm. With the inner end of the catheter placed near the os, a glass pipette is inserted into the outer end and the semen withdrawn by suction. The amount of material recovered is small; generally about one to three tenths cc., and consists of the secretions of the testes, epididymis, seminal vesicle, prostate and Cowper's glands from the male. These may also be mixed with uterine cervical and vaginal secretions, also lymph, epithelial cells, and leucocytes from the female.

During the earlier stages of the work, a female was simply bred once to a male for the recovery of semen without previously attempting to remove any uterine or vaginal secretions which might be present. As the work progressed, it was considered advisable to remove as much as possible of these secretions from the female, in the manner described above, before breeding her, and this step was worked into the routine of our practice. In the great majority of cases the amount of these secretions thus recovered before the breeding was negligible, but sometimes as much as 0.8 cc. was withdrawn. This material differs much in physical properties from semen. It is generally thicker, more viscous, yellowish in color, and does not mix freely with a saline fluid (Ringer's), but gives a mixture with two phases. It is heavily loaded with leucocytes and other cells. Specimens of semen which are mixed in appreciable amounts with this material are easily recognized, and were discarded for study of numbers of sperm per cubic millimeter.

BEHAVIOR OF MALES

The act of coition is of very short duration in the rabbit—one or two seconds suffices. The male generally mounts the female at once; when she is in heat she will raise her hinder parts and elevate the vulva. By a single thrust of the male the act is completed, and the force of the thrust varies with different males.

The time interval between services is influenced by many factors; the following circumstances tend to prolong the service interval; a high temperature in the laboratory; indifference of the female; heavy service by the same male a few days previously; a large number of copulations on the given day; allowing the female and male to remain together in the same cage after coition; failure to furnish 'fresh' females after the male has served the same operated animal several times; failure on the part of the experimenter to crowd the male to his maximum. The time interval between services ranged from five minutes in the early part of a given series to as much as twenty minutes in the latter part of the series. The great majority of services were made at intervals of ten minutes.

Twenty services in the space of about three hours is not the utmost that a rabbit can do; in our own work they have occasionally made several more. However, this was chosen as a safe upper limit, for on many more occasions we have been unable to induce the male to perform as many as twenty services in an afternoon, there being in these cases a complete temporary loss of desire. Several days were required for males to fully recuperate from a long series of services. It was the aim to allow at least seven days' rest between heavy-service days. When such series of matings follow each other too closely the males were unable to copulate even though they made pronounced efforts.

VOLUME OF SEMEN RECOVERED

The greater mass of the seminal fluid is made up by secretions from the accessory glands. These are true secretory glands, and if their behavior is analogous to other glands of this class,

they will show reduced activity after continued stimulation. In fact, workers for the most part agree that reduced volumes of semen is one of the most regular concomitants of excessive sexual activity.

Lewis ('11, p. 30) recovered 65 cc. from a stallion on the first copulation of a test. This same stallion then made one copulation daily for nine days. On the ninth day only 5 cc. of semen were recovered. Again after the horse had rested nineteen days, 60 cc. of semen were recovered from a single copulation.

Iwanoff ('07, p. 494) found that the volume of semen ejected by stallions generally decreases with several copulations, but this decrease is variable. He reports one case of a stallion in which 33 cc. was discharged at the first copulation, 30 cc. on the second, and 28 cc. on the third copulation on the same day. The day following 20 cc. were discharged on the first copulation and 18 cc. on the third. On the third day there were 30 cc. on the first, 25 cc. on the second, but 40 cc. on the fourth copulation.

In a single observation reported by Lode ('91) in man, the volume of semen decreased from 3000 cu.mm. on the first ejaculation to 2000 cu.mm. on the second copulation of the day. On the whole, however, Lode's results are in remarkable contrast to those mentioned above. In the case of a dog the total volume recovered from the first ejaculation was 75. cu.mm. and on the fourth ejaculation of the day 1500 cu.mm. On a second observation, the volume of the first ejaculation was 800 cu.mm. and 1500 on the fourth (the length of the interval between these ejaculations is not stated). Lode makes much of this point; he quotes Ludwig ('91) as saying that "Man vermuthet, dass eine öftere Entleerung des Samen die Neubildung desselben beschleunige," and Nothnagel ('91), "Manches weist vielmehr darauf hin, dass nur nach vorherigen Entleerungen eine stärkere Production stattfindet."

The volume of semen discharged by the male rabbit is necessarily very small, since the animal is small and the time of ejaculation is extremely short. We have found it impossible, as has

TABLE 1

Volume of semen recovered from different services in cubic centimeters

| DATE | INTERVAL SINCE LAST SERVICE | 1ST | 5TH | 10TH | 15TH | 20TH |
|---------------|-----------------------------------|-------|-------|-------|-------|-------|
| <i>Male 1</i> | | | | | | |
| <i>1916</i> | <i>days</i> | | | | | |
| 6-28 | 10 | 0.225 | | 1.01 | | |
| 6-30 | 2 | 0.295 | 0.460 | 0.645 | | |
| 7- 6 | 6 | | | 0.12 | | |
| 7-13 | 7 | 0.435 | 0.209 | 0.165 | | |
| 8-23 | 13 | 0.560 | 0.105 | | 0.05 | |
| 8-28 | 5 | | 0.05 | | | |
| 11-14 | 7 | | | | 0.34 | |
| 11-18 | 4 | | | 0.1 | | |
| 12- 9 | 6 | | | | 0.3 | |
| 12-22 | 3 | 0.071 | 0.210 | | | |
| 12-26 | 4 | 0.245 | | | 0.095 | |
| <i>1917</i> | | | | | | |
| 1- 1 | 6 | 0.19 | | | | 0.09 |
| 1- 4 | 3 | 0.600 | | | | |
| 1-13 | 9 | | | | 0.05 | 0.04 |
| 1-14 | 1 | 0.300 | | | | |
| 6-13 | 73 | 0.714 | 0.107 | 0.10 | 0.08 | 0.05 |
| 6-18 | 5 | 0.34 | | | | |
| 6-29 | 11 | 0.512 | | 0.205 | | 0.175 |
| 7- 9 | 10 | 0.575 | | 0.072 | 0.085 | 0.13 |
| 7-16 | 7 | 0.515 | | 0.170 | 0.145 | 0.07 |
| <i>Male 3</i> | | | | | | |
| <i>1916</i> | | | | | | |
| 7- 7 | 11 | 0.2 | 0.2 | | | |
| 7-11 | 4 | 0.230 | | 0.15 | | |
| 9-16 | 29 | 0.190 | 0.09 | 0.05 | 0.05 | |
| 9-22 | 6 | 0.500 | | | | |
| 9-26 | 4 | 0.500 | | | | |
| 10-22 | 16 | 0.400 | 0.30 | 0.30 | 0.3 | |
| 11- 6 | 15 | 0.300 | | | | |
| 12-21 | 31 | 0.499 | 0.715 | 0.21 | | |
| 12-30 | 9 | 0.074 | 0.4 | | | |
| 12-31 | 1 | 0.200 | | | 0.05 | |
| 1-24 | 24 | 0.200 | | | | 0.13 |

TABLE 1—Continued

| DATE | INTERVAL SINCE LAST SERVICE | 1ST | 5TH | 10TH | 15TH | 20TH |
|--|-----------------------------------|----------|--------|--------|--------|--------|
| <i>Male 4</i> | | | | | | |
| <i>1916</i> | <i>days</i> | | | | | |
| 6-27 | | 0.460 | 0.214 | 0.015 | | |
| 6-29 | 2 | 0.25 | | | | |
| 7- 8 | 9 | 0.212 | | | | |
| 11- 9 | 25 | 0.610 | 0.07 | | | |
| 12-28 | 11 | 0.1 | | 0.1 | | |
| <i>1917</i> | | | | | | |
| 1-10 | 13 | | | | | 0.13 |
| 1-19 | 9 | | | | | |
| 6-14 | 17 | | 0.09 | | | |
| <i>Male 5</i> | | | | | | |
| <i>1917</i> | | | | | | |
| 6-15 | | 0.28 | 0.05 | 0.13 | 0.05 | |
| 6-28 | 13 | 0.12 | | 0.163 | | 0.082 |
| 7-13 | 15 | | 0.14 | | 0.145 | 0.08 |
| Means | | 0.341 | 0.213 | 0.218 | 0.134 | 0.098 |
| Standard deviation of means | | ±0.03072 | 0.0436 | 0.0593 | 0.0287 | 0.0122 |
| Standard deviation of difference of means | | ±0.0533 | 0.0735 | 0.0658 | 0.0311 | |

already been pointed out, to recover the entire amount of the discharge, neither is it possible by the methods used to recover the semen free entirely from the secretions of the female genital tract. But there is no a priori reason to think that the amounts recovered are not proportional to amounts deposited. To measure such small amounts of a thick, viscous fluid, volumetric methods are not feasible, and the record of amounts was, in all but a few cases, obtained by weighing.

The results obtained are shown in table 1. The decrease in volume with advanced services shown by these figures is certainly not pronounced. The means from the different end services and standard deviations of these means are given. The standard deviations of the differences between the means (calculated

by the formula $\sigma x - y = \sqrt{\sigma x^2} + \sigma y^2$ where x and y represent two different means) are also set down. If an observed difference between two means exceeds three times the standard deviation calculated thus, then the difference can hardly be ascribed to fluctuations of sampling (Yule, p. 346, also 140, Ed. 1916). The means, to be sure, show a continuous decline, but the differences between no two contiguous means, as tested by the above principle, are significant.

If we consider the differences in mean volumes between the 1st and the 15th service, however, we have a decrease for the 15th which is certainly significant. The standard deviation for the difference between the 1st and the 15th means is 0.0420, only about one-fifth the difference between the means. The corresponding figure for the 1st-20th is 0.0329 which is less than one-seventh of the difference between the means. In a very general way our data on volume of semen corroborate the work of others cited with the horse and with man, but it is not to be compared, in significance, with the results quoted on horse, man, and dog, in which cases, the entire discharge may be recovered and in which additions from the female tract may be avoided.

CHANGES IN GENERAL PROPERTIES

Certain changes in general properties of the semen occur when a number of coitus rapidly succeed each other. The semen becomes less viscous and tends to lose its characteristic milky appearance until at the 20th service the fluid recovered is frequently of a thin, watery nature. In other words, under continued stimulation the glands which produce the semen not only show reduced activity, but the quality of their product is changed as well. This change in consistency and composition of semen due to excessive sexual activity has been recorded by other workers. Lode ('91) found that in the case of the dog there was a decrease in specific gravity of semen from 1.014 at the first ejaculation to 1.010 on the third ejaculation in a day, and Lewis ('11, p. 37) reports a decrease in total solids after a stallion had served twice daily for a number of days.

NUMBER OF SPERM PER UNIT VOLUME

A few investigators have made counts of the number of sperm per unit volume of semen recovered after the male has engaged in little or much sexual activity. Conspicuous among these is Lode, who was the first to attempt a precise numerical determination of the sperm content of mammalian semen. He presents two observations on man; in the first case the number of spermatozoa per cubic millimeter decreased from 53,200 at the first copulation to 0 on the third copulation of that day. On a second observation, the number decreased from 56,800 on the first to 19,400 on the second copulation. With a dog, the number of sperm for the first ejaculation was 75,000 per cubic millimeter and fell to 3008 on the fourth ejaculation. With a second observation on the same dog, the numbers were 50,000 on the first ejaculation and 29,000 on the fourth. Still a third count at a later period showed 56,840 on the first and no sperm on the fourth ejaculation. Mantegazza states (Stigler, '14) that in semen of man obtained from the second ejaculation in one hour there were scarcely one-half as many sperm in the same volume as in semen from the first ejaculation.

Iwanoff ('07, p. 494) in writing of a stallion, says that the number of sperm cells decreased greatly during the third and fourth copulations in a day, but he does not give the numbers. Lewis ('11) reports the number of sperm cells per cubic millimeter in the semen from a draft stallion as 131,750 at the first service and 5840 on the ninth service made at the rate of one copulation daily for nine successive days. Another stallion showed 68,500 sperm cells per cu.mm. in semen from the third copulation made in two days and 23,000 per cu.mm. in the twentieth copulation made at the rate of two copulations daily.

The counts reported from our own work do not represent absolute number of sperm per cubic millimeter, but a close approximation to the same, based upon the assumption of 1.0 as the specific gravity of semen. As mentioned, volumetric methods with the very small quantities of this viscous fluid available are not suitable for quantitative work. In practice the whole

TABLE 2

Number of sperm per cubic millimeter in semen from different services

| DATE | INTERVAL SINCE LAST SERVICE | 1ST | 5TH | 10TH | 15TH | 20TH |
|---------------|-----------------------------------|---------|--------|--------|--------|--------|
| <i>Male 1</i> | | | | | | |
| <i>1915</i> | <i>days</i> | | | | | |
| 11-13 | | 244,000 | | | | |
| 11-17 | 4 | 344,000 | | | | |
| <i>1916</i> | | | | | | |
| 6-22 | 4 | 270,000 | | | | |
| 6-28 | 6 | 5,000 | | | | |
| 6-30 | 2 | 220,000 | 78,000 | 25,000 | | |
| 7- 6 | 37 | 93,700 | 27,000 | 13,000 | | |
| 7-13 | 7 | 126,000 | 20,750 | 2,500 | | |
| 8- 9 | | | | 1,250 | | |
| 8-23 | 14 | 59,700 | 37,000 | | | |
| 9-16 | 15 | 31,000 | 26,000 | 24,000 | 17,857 | |
| 10-13 | 11 | 7,140 | | | | |
| 11-25 | 2 | 6,000 | | | | |
| 12- 9 | 6 | | | | 800 | 250 |
| 12-22 | 3 | 62,000 | 0 | | | |
| 12-26 | 4 | 72,000 | | | 440 | |
| 1- 1 | 6 | 194,000 | | | | 12,000 |
| 1- 4 | 3 | 38,000 | | | | |
| 1-13 | 9 | | | | 1,120 | |
| 1-14 | 1 | 22,000 | | | | |
| <i>1917</i> | | | | | | |
| 6-13 | 43 | 120,000 | 6,000 | 41,000 | 9,000 | 5,000 |
| 6-18 | 5 | 57,000 | | | | |
| 6-29 | 11 | 140,000 | | 4,000 | | 8,000 |
| 7- 9 | 10 | 230,000 | | 6,000 | 400 | 700 |
| 7-16 | 5 | 109,000 | | | 720 | 200 |
| <i>Male 3</i> | | | | | | |
| <i>1915</i> | | | | | | |
| 12- 8 | | 235,000 | | | | |
| <i>1916</i> | | | | | | |
| 7- 3 | 8 | 21,250 | | 55,000 | | |
| 7- 7 | 4 | 108,000 | | 13,600 | | |
| 7-11 | 4 | 279,000 | | | | |
| 9-26 | 30 | 6,250 | | | | |
| 10-22 | 16 | 7,290 | 43,740 | 26,250 | 10,410 | |
| 11- 6 | 15 | 66,700 | 16,300 | | | |
| 12-21 | 30 | 10,786 | 10,700 | 29,000 | | |
| 12-30 | 9 | 44,000 | 72,000 | | 300 | |
| 12-31 | 1 | 47,000 | | | | |
| <i>1917</i> | | | | | | |
| 1-24 | 24 | 26,000 | | | | |

TABLE 2—Continued

| DATE | INTERVAL SINCE LAST SERVICE | 1ST | 5TH | 10TH | 15TH | 20TH |
|---|-----------------------------------|---------|---------|--------|-------|-------|
| <i>Male 4</i> | | | | | | |
| 1916 | | | | | | |
| 6-27 | 10 | 230,000 | 50,000 | | | |
| 6-29 | 2 | 3,000 | | | | |
| 7- 8 | 9 | 191,400 | 117,500 | | | |
| 9-30 | 8 | 8,330 | | | | |
| 10- 1 | 1 | | | | | |
| 11- 9 | 24 | 13,000 | 5,000 | 1,300 | | |
| 1917 | | | | | | |
| 1-10 | 20 | 216,000 | | | | |
| 1-19 | 9 | | | | | 107 |
| 6-14 | 30 | | 13,000 | | | |
| <i>Male 5</i> | | | | | | |
| 6-15 | | 115,000 | 35,000 | 3,000 | 500 | |
| 7-13 | 28 | | 1,360 | | | 1,680 |
| Means..... | | 104,578 | 34,886 | 14,692 | 4,155 | 3,492 |
| Standard deviations of means..... | | 15,318 | 6,991 | 3,784 | 1,840 | 1,458 |
| Standard deviations of difference of means..... | | 16,836 | 7,949 | 4,206 | 2,346 | |

mass of semen recovered was placed into a weighed dish. The weight of semen present was multiplied by 9 and this volume of diluent (Ringer's isotonic solution) was added. In this shape the specimen was used for study of live sperm. To 1 cc. of the fluid prepared was added 1.5 cc. of a 4 per cent Na_2CO_3 solution (the latter mixed with a small amount of saturated alcoholic methylene blue). This gave (on the assumption of 1.0 as specific gravity of semen) a dilution of 25 times which was found to be most satisfactory for 'normal' semen. The Na_2CO_3 broke up the agglutinated masses and the stain made counting easy when a drop of the fluid prepared thus was placed in the ruled counting chamber of a haemocytometer. We did not determine the actual specific gravity of rabbit semen. Lode made some determinations of this kind on dog and man; for the

former he gives the specific gravity 1.0116 (average of 7 specimens) for the latter 1.035 (average of 9 specimens). It is probable that the specific gravity of rabbit semen is slightly greater than 1.0, and that the figures as published here represent slightly less than the true number of sperm per cubic millimeter.

Table 2 presents the numerical data on sperm content obtained in this experiment. There are a great many gaps in the table and the observations seem badly scattered. This is the result, not of erratic methods, but of the vicissitudes and uncertainties of the work. Oftentimes the males would be used up to the 10th or 15th mating and then the female which had given signs of heat would refuse the service. This was the most usual cause of failure to carry the series through to the limit. Again the male (especially the case with No. 3) would fail to proceed with the series as planned. When there are intervening spaces, such, for example, as with female No. 1, January 1, where records are given only on the 1st and 20th services, the cause of failure to make the readings complete was generally due to one of four causes, a lack of sufficient number of females in heat, the recovery in the semen of urine, of blood, or of perceptible amounts of vaginal or uterine secretions which we had not succeeded in removing before service.

The figures obtained, moreover, show wide and apparently capricious variations. For example, in the case of male No. 1, consider the mating on June 29, 1917. After 11 days' rest the numbers on the 1st and 20th services are 140,000 and 8,000, respectively. On July 9th, after another rest interval of about equal length (10 days), the corresponding numbers are 230,000 and 700. In each case the number at the 20th is greatly reduced, but there is yet a strange lack of harmony in the relation of the numbers for the 1st and 20th services. This somewhat erratic nature of the data may perhaps in a measure be considered as due to the variations in mood and condition of the males, but doubtless in larger measure to the unavoidable imperfections in our methods of recovery of the semen and to the small amounts of the same. That is to say that quantities of female secretions so slight as to be by themselves non-recoverable by catheter, might, when recovered with such small quantities of semen, be

of significance in reducing the sperm counts made upon the same.

The averages of all the readings made from all the males at each service have been determined, and they show an unmistakable downward trend, except that, on account of two unusually large figures in the 20th, the decline from 15th to 20th is less marked than in the other cases. However, as measured by the criterion of three times their standard deviations (with this irregular data the use of the customary probable error is certainly not justified), the differences between contiguous means are without significance except in the case of that between the means for the 1st and 5th. The differences between the means of the 5th and 15th or 20th are also certainly significant, but not that between the 10th and 20th. On the whole the mathematical methods of examining the data indicate that heavy sexual use has no very grave effect upon the number of sperm per cubic millimeter. However, the marked lack of regularity in these data demands that caution be used in accepting the arithmetical constants derived from the data as reliable indices of significance of the means and the data as a whole. In such cases a study of the extended figures themselves may actually give a truer meaning of the facts than the usual statistical methods of analysis. There are 22 cases in table 2 where 2 or more counts were made within a single series of matings. In 16 of these there is a marked falling off in sperm content of the semen as the number of services increases; in three cases there is a tendency for an increase of like figures and in three cases there is neither a clear-cut upward or downward trend of the counts as service number increases. It seems to the writers that by reading across the horizontal rows, one after another, one is furnished with almost convincing proof that there is a well-marked reduction in number of sperm per cubic millimeter in the advanced services.

MOTILITY

Motility of sperm cells has attracted as much or perhaps greater interest than the matter of their number. Motion is effected largely by a rapid vibration of the tail piece, but in some cases it appears that a spiral rotation of the entire body may aid

progress. The sperm is oriented by currents which exist in the genital tract, as Kraft showed in 1890 (p. 216). He flooded a piece of uterine mucous membrane from a freshly killed cow with an isotonic salt solution and when rabbit sperm were placed thereon, they were observed to swim rapidly against the currents set up by the cilia of the membrane. Roth recorded a similar observation in 1893 (p. 352).

Pronounced motility may be in most cases accepted as evidence of the fertility of the male, but this is not always the case. For example, Detlefsen ('14, pp. 91-93) found in case of his hybrid guinea-pig males that though the presence of a high percentage of normal active spermatozoa in the fluid obtained from the epididymis was as a rule a very good indication of breeding power, nevertheless 10.2 per cent of such hybrid guinea-pig males were completely sterile though a copious supply of active sperm was present. And Reynolds ('16), after extensive studies on the question of sterility in the human subject, insists that the mere presence of active spermatozoa in the semen of a man is by no means certain evidence of his power to reproduce. He considers that investigations as to fertility, must take into account not only the existence of motility, but the duration of motion and still more the quality of motion present.

In regard to quality of motion, several writers have observed that various categories may be made out. As long ago as 1856, Kölliker in his description of the stimulus which certain substances produce in mammalian sperm, distinguished between an 'axedrehung' and a 'Schlange bewegung,' and Iwanoff ('07) emphasizes the importance of 'mouvements progressifs' in contrast to 'mouvements vibration.' But Reynolds' ('16) observations have been much more extensive and his interpretations more suggestive than those of other writers and his work deserves further mention. Reynolds' studies are based upon the examination of 45 specimens of semen from man collected sometimes free from the secretions of the female and sometimes mixed with these secretions. His studies have led him to the 'positive differentiation' of 5 types of motion, 3 of which he considers as

normal, and they appear to be consecutive phases. 1. 'Progressive Vibratile' motion consists of a rapid vibration of the after part of the flagellum propelling the sperm rapidly forward with the head moving practically in a straight line. The direction, in the absence of currents, is random. This type of motion would be well suited for a prolonged journey to the Fallopian tube. 2. 'Undulatory tactile' which normally follows type 1 is characterized by a slow lashing from side to side of the entire tail, causing the head and middle piece to sway back and forth through an arc of sometimes 90 degrees. Only slight linear advance is made in this phase and the path is exceedingly devious. Having arrived at the tube this kind of movement would provide that the sperm 'find' the egg. The sperm at this stage seem to be negatively thigmotactic, having the power to touch and back off from other bodies. 3. The third type succeeds the second and is distinguished by a 'bunting' action of the head and a rapid vibratile motion of the tail when in contact with another body. Reynolds considers that this type enables the spermatozoa to penetrate the egg membranes and to fertilize the ovum. The types of motion which are said to be abnormal will not be considered here.

In our own work we have attempted to consider the type of motion present as well as the rate of motion, but we find it exceedingly difficult to make definite statements in regard to this matter. In a very general way our observations are in harmony with those of Reynolds, i.e., we find that the specimens of semen which show the largest amount of progressive vibratile movement as a rule show the longest duration of motion. But with the semen handled as herein described the essential cyclic nature of the different phases of motion as described by Reynolds is not clearly brought out; for example, even in the sperm of lowest 'vitality' as measured by duration of motion, 'stationary bunting' is the most usual type of motion. Further reference to type of motion will be made in the next section.

RATE OF MOTION

Very few direct observations on the rate of motion of spermatozoa have been made. Bischoff ('42) reports that Henle observed mammalian sperm to move forward in a straight line at the rate of about 0.056 mm. per second. Lott ('72) found that sperm taken from the epididymis of the dog move at the rate of 0.06 mm. per second against a current under a glass slide. Our own work, as will be shown later, indicates a velocity for normal rabbit sperm of about 0.05 mm. per second.

Many observations have been made, however, on the least time interval between the deposit of the semen and its arrival into the upper genital tract. Heape ('05) finds that as a rule sperm of rabbit are to be found at the top of the uterus two hours after copulation and within the folds of the infundibulum two hours later. Coste ('69, quoted by Hensen) states that in the rabbit, sperm are found at the ovaries two and three-quarter hours after copulation. The linear distance from cervix to fimbriae in the rabbit is about 280 mm. But if we wish to estimate the length of the path by which a sperm would cover this total distance by its own motile activity unaided by any other force, we should, on account of its devious lateral meanderings and the intricate folds of the surface over which it must travel, multiply the distance by at least 2. If we now allow three hours as the required time for this journey, the sperm would move at just about 0.05 mm. per second.

Data given by Payne ('14) for the chicken show unusually quick action. He killed virgin pullets at varying intervals after breeding and reports finding sperm at the extreme upper end of the shell gland, a distance of about 650 mm. from cloaca, in one and one-half hours after breeding. Again, if we multiply this distance by 2 and if the sperm travel this distance unaided by other forces, the rate would be 0.24 mm. per second. This is about four times as fast as any direct determinations which have been reported. Obviously, some other force, such as the aspirations of the os and peristalsis of the upper tract (Bischoff, '42, Lott, '72, Heape, '98), is operating, and the interval elapsing

between the deposit of the sperm and the time of its arrival in the upper tract cannot be used in reckoning the rate at which the sperm may move by virtue of its own motive power.

The point on which the present work particularly bears is the influence on the rate of motion, of unusual sexual exercise by the male. Iwanoff has reported data which bear on this point. He ('07, p. 494) mated a stallion which was known to produce sperm of great activity as follows: Beginning August 20, this stallion made two copulations, and August 23, four copulations at intervals of two hours. Sperm from the last three copulations showed very feeble motion (numerical data not given). Stigler ('14, p. 219) states that Mantegazza obtained semen from a man one-half hour after a previous copulation and that the sperm in the same 'mit viel geringer Energie bewegten.'

In making the counts given in this paper the semen was in all cases diluted with 9 volumes of Ringer's solution. In undiluted rabbit semen the sperm are so crowded that a straight-ahead course can be pursued for only a very short distance and the motion is greatly impeded. Moreover, the varying viscosities of natural semens, as Lepinase ('17) states, serve as an external factor to modify the rate of motion. A drop of this diluted semen was placed in the counting chamber of a haemocytometer. This instrument is very well suited for such readings because the graduations make the linear distance covered by the sperm easily determined and there is an abundance of free space in which the cells can move. The observations made on the 'rate of motion' of sperm were always made on individual sperm unencumbered by foreign bodies, showing the progressive vibratile type of motion. A stop-watch was used to determine the time required for a sperm cell to pass over from two to five or six 0.05 mm. spaces on the cytometer. This time interval was set down for a considerable number of sperm on the slide, an average of the results was taken as the correct measure of motility and recorded together with the temperature of the liquid in which the sperm were moving.

Temperature is of course a factor which has a most profound influence upon the rate of motion, and unfortunately this has

TABLE 3

Rate of progressive motion of spermatozoa from different services; millimeters covered per second, and temperature at each observation

| DATE | TEMPERATURE | 1ST | 5TH | 10TH | 15TH | 20TH |
|---------------|-------------|--------------|--------------|--------------|--------------|--------------|
| <i>Male 1</i> | | | | | | |
| 1916 | | mm. per sec. | mm. per sec. | mm. per sec. | mm. per sec. | mm. per sec. |
| 8- 2 | | 0.036 | 0.063 | | | |
| 8- 9 | | 0.066 | 0.065 | 0.038 | | |
| 8-23 | | 0.052 | | | no prog. | |
| 9-16 | 14 | 0.015 | 0.017 | | 0.031 | |
| 10- 2 | 24 | 0.038 | | | 0.022 | |
| 10-13 | 19 | 0.020 | | | | all dead |
| 11-14 | 12 | 0.007 | | | | 0.0008 |
| 11-23 | | | | 0.0042 | | no prog. |
| 12- 9 | | | | | 0.009 | no prog. |
| 12-26 | 20 | 0.038 | | | no prog. | |
| 11-30 | 18 | 0.030 | 0.012 | | | |
| 1917 | | | | | | |
| 1- 1 | 22 | 0.030 | | | | 0.034 |
| 1-13 | 22 | 0.038 | | | | 0.027 |
| 6-13 | 27 | 0.055 | 0.025 | 0.031 | 0.026 | 0.016 |
| 6-18 | 27 | 0.030 | 0.028 | all dead | | |
| 7- 9 | 25 | 0.083 | | 0.042 | no prog. | no prog. |
| 7-16 | 25 | 0.060 | | | | |
| <i>Male 3</i> | | | | | | |
| 1916 | | | | | | |
| 8- 1 | | 0.048 | 0.040 | | | |
| 8-18 | | 0.044 | all dead | | | |
| 1- 6 | 21 | 0.026 | | 0.022 | | |
| 10-22 | 22 | 0.021 | 0.024 | 0.010 | 0.066 | |
| 11- 6 | 22 | 0.029 | | 0.023 | | |
| 12-21 | 21 | | 0.010 | | | |
| 12-30 | 19 | 0.021 | 0.021 | | | |
| <i>Male 4</i> | | | | | | |
| 1916 | | | | | | |
| 10- 1 | no sperm | | 0.023 | 0.009 | | |
| 10-15 | | | 0.004 | 0.016 | | |
| 10-31 | 18 | 0.005 | | no prog. | | |
| 11- 9 | 16 | 0.009 | | 0.012 | | |
| 12-28 | 19 | 0.019 | | 0.008 | | |
| 1917 | | | | | | |
| 6-18 | 21 | 0.017 | | 0.022 | 0.013 | |
| 6-14 | 24 | | 0.035 | | | |

TABLE 3—*Continued*

| DATE | TEMPERATURE | 1ST | 5TH | 10TH | 15TH | 20TH |
|---|-------------|--------|--------|--------|--------|----------|
| <i>Male 5</i> | | | | | | |
| 6-15 | 23 | 0.030 | 0.020 | 0.034 | 0.010 | |
| 6-28 | 27 | 0.025 | | 0.040 | | no prog. |
| Means | | 0.033 | 0.027 | 0.022 | 0.025 | 0.019 |
| Standard deviations of means | | 0.0034 | 0.0049 | 0.0045 | 0.0068 | 0.0062 |
| Standard deviations of differences | | 0.0058 | 0.0067 | 0.0081 | 0.0092 | |

been a factor which we were unable to control. The diluent, glassware, etc., remained at room temperature, which varied widely by days and by seasons, but almost in every case the readings on the various services of a given series were made at temperatures which did not vary more than one or two degrees. Under the circumstances the best that could be done was to record the temperatures at each reading, and these are set down in the table. Unless at least two readings at different services within a given series were obtained they were not set down in the table, consequently many readings on rate which were made are not there included.

The data on rate of motion are given in table 3. Specimens which showed no sperm making progressive vibratile motion although other types of motion were present are recorded as 'no prog.' The means, their standard deviations and the standard deviations of the differences between contiguous means are set down. The slight downward trend of the mean rates as the service number advances, when studied in connection with standard deviations, is seen to be wholly without significance. In every case, except the 1st and 5th, the standard deviation of the difference between the means is actually greater than the differences themselves, whereas if these differences are to be significant the standard deviations should be not more than one-third the latter. These meager results suggest that a certain velocity is characteristic of progressive vibratile motion. If the energy of the sperm becomes spent so that this velocity cannot

be maintained, they rapidly cease this type of motion and become 'bunters' or 'spiral movers.'

But the fact that the velocity of any sperm which are showing progressive vibratile motion is as great in the latter services as in the earlier ones, considered by itself, does not give a true picture of conditions. For in semen from the earlier services the number of sperm which display this type of motion are more numerous. In specimens from the later services it was often necessary to search over numerous fields before a satisfactory number of progressive movers could be observed; in fact, as the table shows in actually one-half of the cases of 20th service, no sperm of this sort were discovered.

The statements quoted from Iwanoff and Stigler to the effect that in semen from heavily taxed males the motion is 'feeble' or shows 'little energy' do represent the general impression that would result from an examination of such material. But it seems this picture is not the result of a slowing down of the *forward* motion so much as the assumption of different types of motion, such as energetic 'bunting' motion, slow undulatory or rotary motion.

DURATION OF MOTION

The duration of motion of spermatozoa both in the female genital tract and outside the body under laboratory conditions has received greater attention from writers than any other phase of study about spermatozoa. Unless sperm show continued activity for several hours within the female genitalia, they never take part in fertilization. The published facts in regard to the length of time sperm may retain their fertilizing power within the body of the female and motility *in vitro* are numerous and not always in harmony with each other, and no attempt will be made to review them here. Semen held *in vitro* will withstand a wide range of temperatures. Mantegazza (Iwanoff, p. 493) asserts that mammalian sperm retain vitality between $-15^{\circ}\text{C}.$ and $47^{\circ}\text{C}.$, and Iwanoff himself carried semen of horse to a point where it solidified (ca. $-15^{\circ}\text{C}.$) without destroying motion when the temperature was raised, but the same author does admit

that a temperature of 100°C . is fatal to the sperm. The length of time that sperm will retain its motility is largely decided by the temperature at which it is held. Lewis found that sperm from the horse retained motility about twice as long as 1°C . as at 30°C . Payne ('14) held sperm from cock birds at 34°F . and 106°F ., and found that those at the former temperature displayed motility three times as long as the latter. Piersol ('93) kept human sperm at 3°C . and ca. 35°C . The former showed motion at the end of nine days, the latter had ceased moving long before. Therefore, in order properly to interpret observations on duration of motion, it is essential that temperature at which observations were made be reported.

In general it may be said that duration of motion is coincident with duration of life of the sperm, but this is not always true. At low temperatures, of course, contractile motion ceases, but returns upon warming. Stigler's ('14) results also show that high temperatures also cause cessation of motion, which may return on cooling. In one case (Versuch, '45) he repeatedly raised (45.5°C .) and lowered (room temperature) the temperature of a specimen of semen, thus causing repeated cessation and return of motion in the specimen. Hensen ('75) reports some very remarkable results which he obtained in inducing action in sperm which has ceased moving, by application of NaOH and KOH solutions. It is probable also that sperm which appear motionless under the microscope may be activated when placed within the female genital tract.

Observations on the effect of heavy sexual service of the male on the duration of motion of sperm produced by him are few. Lewis ('11, p. 30) allowed a heavy draft stallion to make nine consecutive services on as many days. This he considered heavy service for the stallion in question. The results of this series of services on the sperm content of the semen has been discussed. In the semen from the first service 20 per cent of the sperm were alive after 9.5 hours. In the case of 5th service all cells were dead at 9 hours. In case of 6th service 5 per cent were alive after 5 hours, but in the 9th service no cells were alive after 4.5 hours. (All observations at 21°C . to 23°C .) Lewis also offers

TABLE 4

Duration of motion of spermatozoa as affected by number of services. Percentage moving after various periods of time have elapsed. (All semen diluted with ten volumes of Ringer's solution)

| DATE | AVERAGE TEMPERA- TURE | HOURS AFTER RECOVERY | PER CENT ACTIVE | | | | |
|--------|-----------------------------|----------------------------|-----------------|-----|------|------|------|
| | | | 1st | 5th | 10th | 15th | 20th |
| Male 1 | | | | | | | |
| 1916 | | | | | | | |
| 10- 2 | | 16 | 81 | | | 0 | |
| | 24 | 24 | 34 | | | | |
| 10-13 | 19 | 0 | | | | | |
| | | 28 | 80 | | | | |
| | | 48 | 70 | | | | |
| | | | 0 | | | | 0 |
| 10-31 | | 16 | 55 | 2 | | | |
| | | 28 | 45 | | | | |
| | | 44 | 21 | | | | |
| | 18 | 52 | 0 | | | | |
| 11-14 | 19 | 28 | 30 | | | | 0 |
| 12- 9 | 20 | 22 | | | | 0 | |
| 12-22 | | 20 | 0 | 0 | | | |
| 12-26 | | 2 | 98 | | | 35 | |
| | | 4 | 90 | | 40 | 13 | |
| | | 8 | 60 | | 0 | 5 | |
| | | 12 | 33 | | | 0 | |
| | | 16 | 18 | | | | |
| | | 20 | 15 | | | | |
| | | 28 | 1 | | | | |
| | 19 | 36 | 0.5 | | | | |
| | | 48 | 0 | | | | |
| 1917 | | | | | | | |
| 1- 1 | | 0 | 97 | | | | 90 |
| | | 4 | 95 | | | | 50 |
| | | 8 | 40 | | | | 10 |
| | | 16 | 35 | | | | 0 |
| | | 20 | 32 | | | | |
| | | 28 | 10 | | | | |
| | 24 | 32 | 0 | | | | |
| 1-13 | | 0 | 65 | | | 95 | 50 |
| | | 2 | | | | | 38 |
| | | 4 | 58 | | | 44 | |
| | | 8 | 41 | | | 33 | 0 |
| | | 16 | 35 | | | 27 | |
| | | 20 | | | | 20 | |
| | 23 | 24 | 0 | | | 0 | |

TABLE 4—Continued

| DATE | AVERAGE TEMPERA- TURE | HOURS AFTER RECOVERY | PER CENT ACTIVE | | | | |
|--------|-----------------------------|----------------------------|-----------------|-----|------|------|------|
| | | | 1st | 5th | 10th | 15th | 20th |
| Male 1 | | | | | | | |
| 1917 | | | | | | | |
| 6-13 | | 0 | 90 | | 90 | | 50 |
| | | 8 | 80 | 80 | 40 | 35 | 10 |
| | | 16 | 2 | 0 | 0 | 0 | 2 |
| 6-18 | 25 | 20 | 2 | | | | |
| | | 0 | 45 | 60 | | | |
| | | 4 | 45 | 35 | | | |
| 6-29 | 27 | 12 | 5 | 2 | | | |
| | | 0 | 72 | | 55 | | 55 |
| | | 4 | 60 | | 40 | | |
| | | 8 | | | 35 | | 50 |
| | | 12 | 50 | | 10 | | 15 |
| | | 20 | 1 | | 0 | | 0 |
| 7- 9 | | 0 | 80 | | 60 | 20 | 85 |
| | | 4 | 50 | | | 0 | |
| | | 8 | 40 | | 35 | | 35 |
| | | 12 | 35 | | 30 | | 25 |
| | | 20 | 20 | | 0 | | 30 |
| | | 24 | 10 | | | | 10 |
| 7-16 | 29 | 28 | 0 | | | | 0 |
| | | 0 | 90 | | 75 | | 75 |
| | | 4 | 80 | | 50 | | 30 |
| | | 8 | 35 | | 25 | | 0 |
| | | 12 | 35 | | 25 | | |
| | | 24 | 10 | | 0 | | |
| Male 3 | | | | | | | |
| 1916 | | | | | | | |
| 10- 6 | | 16 | | 0 | | | |
| | | 20 | 50 | | | | |
| 10-22 | | 24 | 37 | 90 | 83 | 20 | |
| | | 40 | 35 | 77 | 38 | 0 | |
| | | 44 | | 30 | 16 | | |
| | | 48 | 2 | | | | |
| | | 18 | 56 | 0 | 0 | | |
| | | | 20 | 0 | | 10 | |
| 11- 6 | 22 | 34 | | | 0 | | |

TABLE 4—Continued

| DATE | AVERAGE TEMPERA- TURE | HOURS AFTER RECOVERY | PER CENT ACTIVE | | | | |
|--------|-----------------------------|----------------------------|-----------------|-----|------|------|------|
| | | | 1st | 5th | 10th | 15th | 20th |
| Male 3 | | | | | | | |
| 1916 | | | | | | | |
| 12-21 | 19 | 4 | 0 | | 80 | | |
| | | 16 | | | 13 | | |
| | | 20 | | | 0 | | |
| 12-30 | 22 | 4 | 10 | 35 | | | |
| | | 16 | 0 | 25 | | | |
| | | 24 | | 0 | | | |
| Male 4 | | | | | | | |
| 10- 1 | 22 | 20 | | 72 | 0 | | |
| | | 24 | | 13 | | | |
| | | 28 | | 1 | | | |
| 10-15 | 19 | 28 | 0 | 75 | 40 | | |
| | | 50 | | 0 | 0 | | |
| 11- 9 | 17 | 20 | 75 | | 23 | | |
| | | 24 | 29 | | | | |
| | | 40 | | | 0 | | |
| 12-28 | 17 | 44 | 0 | | | | |
| | | 4 | 35 | | 50 | | |
| | | 10 | | | 18 | | |
| | | 16 | 5 | | 15 | | |
| | | 20 | 0 | | 14 | | |
| | | 28 | | | 2 | | |
| 1-10 | 17 | 36 | | | 0 | | |
| | | 0 | 71 | | 77 | 80 | |
| | | 4 | 66 | | 30 | | |
| | | 8 | | | 18 | 15 | |
| | | 12 | 45 | | 0 | 15 | |
| | | 16 | 40 | | | 0 | |
| 6-14 | 17 | 20 | 40 | | | | |
| | | 24 | 19 | | | | |
| | | 28 | 0 | | | | |
| | | 0 | | 55 | | | |
| | | 4 | | 30 | | | |
| | | 12 | 24 | 0 | | | |
| Male 5 | | | | | | | |
| 6-15 | 17 | 0 | 25 | 50 | 90 | 95 | |
| | | 4 | 10 | 50 | 40 | 75 | |
| | | 8 | 10 | 40 | 20 | 60 | |

TABLE 4—*Concluded*

| DATE | AVERAGE TEMPERA- TURE | HOURS AFTER RECOVERY | PER CENT ACTIVE | | | | |
|--------|-----------------------------|----------------------------|-----------------|-----|------|------|------|
| | | | 1st | 5th | 10th | 15th | 20th |
| Male 5 | | | | | | | |
| 1916 | | | | | | | |
| 6-15 | 24 | 16 | 2 | 0 | 0 | 2 | |
| | | 24 | 1 | | | 2 | |
| | | 28 | 0 | | | 1 | |
| 6-28 | | 0 | 85 | | 30 | | 30 |
| | | 4 | 70 | | 20 | | 15 |
| | | 8 | | | 10 | | 0 |
| | | 12 | 60 | | | | |
| | | 25 | 20 | 3 | | 0 | |

data from another series of matings with a different stallion in support of the idea that the vitality of the sperm is reduced when the male is overtaxed but this case is not so demonstrative. Stigler ('14, p. 219) obtained semen from a man two hours after a previous copulation and found that sperm from the latter could successfully resist a high temperature for a longer time than the former. Held at 44.6°C. sperm from the first copulation ceased moving, but remained alive for 1 hour 45 minutes while sperm from the second copulation were killed altogether between 10 and 45 minutes. In our work with the rabbit the duration of motion determinations were made on the specimens prepared for a study of the rate of motion, i.e., the semen was diluted with 9 volumes of Ringer's solution. The conditions under which this work was done do not reduce, but, in fact, as will be shown, prolong the life of the sperm. The parcels of diluted semen were placed into small glass vials with cotton stoppers. These were all placed on the same shelf in the laboratory and were not exposed to direct sunlight, but were exposed to the varying temperatures of the room. On account of this temperature variation the results obtained on different days are sometimes not directly comparable, but the results from a series of specimens obtained within three or four hours of each other may be safely compared among themselves.

Table 4 gives the data on duration of motion, and at the end of each series of observations the 'average temperature' is recorded.

The aim was to make observations at four-hour intervals, but this was approximated in only a very general way; in some cases in the earlier part of the work but a single observation 20 hours or so after the semen was secured was recorded. We have discovered no convenient way in which this data may be condensed or summarized beyond the 'note-book' form in which it is given. An average of the first four-hour interval encountered as one passes downward in each 'service' column, at which no sperm are active, suggests itself as an expression of the average duration of motion at each service. But averages calculated in this way conceal the really significant more rapid dropping off in per cent of active sperm which is unmistakable in the semen from high-service groups. The table can best be studied by reading across the rows set opposite each four-hour interval and comparing the per cent of active sperm in semen from the different services at this number of hours after the several samples were taken. For example, in case of male No. 1 on January 1, 1917, the fresh semen (0 hours) from 1st service showed 97 per cent of the sperm active; from 20th only 90. In the next lower row we see that after standing on the table four hours the 1st service semen showed 95 per cent active, the 20th 50 per cent. At 16 hours after recovery there were 35 per cent active from 1st service, but all sperm were inactive in the specimen from the 20th service. But this more rapid decline in per cent active sperm in semen from more advanced service groups is again by no means universal with the data and in some cases the persistence of activity in semen from 15th or 20th service is fully the equal to that from the 1st (Male No. 1, July 9, for example).

A factor which gravely interferes with the precision of these results is that of bacterial growth and its products. No means to use sterile apparatus were at hand, and in spite of scrupulous cleanliness putrefaction of course eventually set in. In a few cases sperm will continue to move even though odors indicate that degenerative processes are well established, but almost always, motion has ceased before bacterial growth has proceeded thus far. Nevertheless sperm may be influenced by the incipient stages of putrefaction in the fluid and the onset of this process was not under our control.

TABLE 5

Duration of motion-'natural' semen compared with 'diluted'

| DATE | HOURS AFTER RECOVERY | PER CENT ACTIVE | | | | | |
|-------|----------------------------|-----------------|---------|---------|---------|---------|---------|
| | | 1st | | 5th | | 10th | |
| | | Natural | Diluted | Natural | Diluted | Natural | Diluted |
| 10-13 | 28 | 0 | 70 | | | | |
| | 48 | | 0 | | | | |
| 11-14 | 28 | 15 | 30 | | | | |
| 10-31 | 16 | 50 | 55 | | | | |
| | 28 | 40 | 45 | | | | |
| | 44 | 0 | 21 | | | | |
| | 52 | | 0 | | | | |
| 10- 6 | 20 | 30 | 50 | | | | |
| 10-22 | 24 | 0 | 37 | 22 | 90 | 20 | 83 |
| | 44 | | | 5 | 30 | 0 | 16 |
| 10- 1 | 20 | | | 0 | 72 | | |
| 10-15 | 28 | 0 | 0 | | | | |
| 11- 9 | 20 | 58 | 75 | | | | |
| | 24 | 21 | 29 | | | | |

However, we feel that a study of the data as above suggested will leave one with a decided opinion that there is a well-marked tendency for the sperm in semen from the higher-service groups to show less 'vitality' or 'potential energy,' as measured by the duration of motion which they will display.

Table 5 is inserted here to compare the duration of motion of sperm in semen as it was recovered from the female with sperm in semen diluted with ten volumes of an isotonic solution. The counts of 'per cent active' are much less accurate with undiluted semen than with diluted because of the crowding of the sperm. Moreover, they are so close together that the agitation set up by one active individual may cause several others to appear active, whereas they are not actually of themselves motile. But the point at which all sperm are inactive may of course be determined with as great accuracy in one case as in the other.

Though the table is not extensive, the deduction to be made from these figures is plain: namely, that sperm in diluted semen display a longer duration of motion than sperm in 'natural' semen. The probable cause of this phenomenon is that in the

TABLE 6

Matings and resulting pregnancies from the various end services

| MALE NUMBER | 1st | | | 5th | | | 10th | | | 15th | | | 20th | | |
|-------------|---------------|-------------|----------------------|---------------|-------------|----------------------|---------------|-------------|----------------------|---------------|-------------|----------------------|---------------|-------------|----------------------|
| | Total matings | Pregnancies | Per cent pregnancies | Total matings | Pregnancies | Per cent pregnancies | Total matings | Pregnancies | Per cent pregnancies | Total matings | Pregnancies | Per cent pregnancies | Total matings | Pregnancies | Per cent pregnancies |
| 1 | 17 | 11 | 64.70 | 9 | 7 | 77.77 | 12 | 6 | 50.00 | 12 | 6 | 50.00 | 26 | 10 | 38.46 |
| 3 | 14 | 12 | 85.71 | 15 | 9 | 60.00 | 11 | 6 | 54.54 | 5 | 2 | 40.00 | | | |
| 4 | 12 | 8 | 66.66 | 12 | 6 | 50.00 | 12 | 6 | 50.00 | 12 | 4 | 33.33 | 19 | 6 | 31.57 |
| Total | 43 | 31 | 72.09 | 36 | 22 | 61.11 | 35 | 18 | 51.42 | 29 | 12 | 41.37 | 45 | 16 | 35.55 |

natural semen the by-products of the metabolism of the sperm, and also of the developing bacteria more quickly reach a concentration that is deleterious or even fatal to the sperm. Whether the fertilizing power of the sperm is diminished by this dilution is a matter for further study. Certainly it is not completely destroyed, for Iwanoff reports inseminating rabbits with semen diluted with a weak Na_2CO_3 solution, with resulting pregnancy.

CERTAINTY OF PREGNANCY

In the previous sections of this paper we have shown that excessive sexual service causes decrease in amount of ejaculated semen, decrease in number of sperm cells per cubic millimeter, decrease in the proportion of sperm that show progressive motion, and decrease in duration of motion. All these changes are such as to reduce the likelihood of such semen causing pregnancy of the female. In other words, we should expect the percentage of pregnancies induced by the copulations to become less as the number of preliminary services increases. This is in truth found to be the case as shown in table 6.

The decline proceeds with fair regularity whether we consider the males separately or together. This reduction in the per cent of effective matings when the male is sexually overworked is recognized by those engaged in animal breeding as one of the

TABLE 7
Size of litters at different services

| MALE NUMBER | 1ST | | 5TH | | 10TH | | 15TH | | 20TH | |
|---------------|------------------------|----------------------|------------------------|----------------------|------------------------|----------------------|------------------------|----------------------|------------------------|----------------------|
| | Num- ber litters | Aver- age size | Num- ber litters | Aver- age size | Num- ber litters | Aver- age size | Num- ber litters | Aver- age size | Num- ber litters | Aver- age size |
| 1 | 6 | 6.66 | 6 | 6.00 | 7 | 7.71 | 7 | 5.85 | 11 | 4.27 |
| 3 | 11 | 6.50 | 8 | 5.75 | 7 | 7.57 | 2 | 10.5 | | |
| 4 | 8 | 7.75 | 6 | 6.16 | 6 | 5.33 | 4 | 5.5 | 6 | 5.00 |
| All males.... | 25 | 6.92 | 20 | 5.95 | 20 | 6.95 | 13 | 6.46 | 17 | 4.53 |

most noticeable and universal concomitants of heavy sexual service, but there is great divergency of opinion as to how frequent or how many copulations constitute 'heavy service' with various classes of domestic animals. Apparently in bringing about twenty services in three or four hours we have approached a point which for the rabbit seriously curtails his ability to produce offspring, and furthermore we have demonstrated in large part what is the direct basis for this curtailment.

SIZE OF LITTER

Having observed the described changes in the vital properties of the sperm in semen from advanced services, and also what is probably in large part a result of the same, namely, the reduced likelihood of pregnancy resulting from the more advanced services, it would seem a priori to follow that the number of young per litter would likewise undergo a reduction as the number of copulations increases. Table 7 presents the facts in regard to this matter.

Inspecting the columns up and down reveals no superiority of one male over another. Inspecting the horizontal rows reveals no marked effect of number of copulations on litter size; in fact, it may be questioned if it reveals any significant reduction. Certainly any falling off in litter size up to the 15th service is impossible to detect by inspection, but a very perceptible drop occurs between the 15th and 20th service.

TABLE 8

Relation between size of litter and number of previous services

| SIZE OF LITTERS | NUMBER OF SERVICES | | | | | |
|-----------------|--------------------|-----|------|------|------|-------|
| | 1st | 5th | 10th | 15th | 20th | Total |
| 1 | | 1 | 1 | 2 | 1 | 5 |
| 2 | 1 | 1 | | 2 | 4 | 8 |
| 3 | | 2 | 1 | | 4 | 7 |
| 4 | | | | | 2 | 2 |
| 5 | 3 | 3 | 2 | 1 | 1 | 10 |
| 6 | 7 | 6 | 3 | | | 16 |
| 7 | 6 | 2 | 4 | 1 | 2 | 15 |
| 8 | 5 | 3 | 4 | 2 | 1 | 15 |
| 9 | 1 | | 4 | 2 | | 7 |
| 10 | 3 | 1 | | 2 | 1 | 7 |
| 11 | | 1 | 1 | | 1 | 3 |
| 12 | | | | 1 | | 1 |
| | 26 | 20 | 20 | 13 | 17 | 96 |

Correlation coefficient = -0.2207 ± 0.06526 .

Table 8 shows the complete distribution of the litters on the basis of size arranged as a correlation table. The coefficient of correlation calculated from this table is $-0.2297 - 0.0653$, a figure which, though over three times its probable error, is yet of rather questionable significance. An inspection of the table, however, shows up a marked preponderance of small litters in the 20th service. If it is true that the critical number of services, so far as decreasing the number in the litter is concerned, is between the 15th and the 20th service, then of course the above method of determining the correlation between frequency of copulation and number in litter is not valid, for in this case the 20th, as one group, should be compared with all others as another group, and not be considered as one of five coordinate groups. In table 9 the first four columns of table 8 are combined and the percentage of the total litters in each group which are of a given size calculated.

The preponderance of small litters in the 20th-service group is thus revealed. If we condense the data still further into a four-fold classification, we find that in the 1 to 15 group 25.32 per cent

TABLE 9

Size of litter in 1st to 15th service group compared with 20th

| SIZE OF LITTER | 1-15 | | 20 | |
|----------------|------------------------------------|--------------------------------------|------------------------------------|--------------------------------------|
| | Number of litters of given size | Per cent of litters of given size | Number of litters of given size | Per cent of litters of given size |
| 1 | 4 | 5.06 | 1 | 5.88 |
| 2 | 4 | 5.06 | 4 | 23.53 |
| 3 | 3 | 3.80 | 4 | 23.53 |
| 4 | 0 | 0 | 2 | 11.76 |
| 5 | 9 | 11.39 | 1 | 5.88 |
| 6 | 16 | 20.25 | 0 | |
| 7 | 13 | 16.45 | 2 | 11.76 |
| 8 | 14 | 17.72 | 1 | 5.88 |
| 9 | 7 | 8.86 | 0 | 0 |
| 10 | 6 | 7.59 | 1 | 5.88 |
| 11 | 2 | 2.52 | 1 | 5.88 |
| 12 | 1 | 1.28 | 0 | 0 |
| Total..... | 79 | | 17 | |

of the litters were five or less in size, whereas in the 20th-service group 70.58 per cent of the litters were five or less in size. In spite of the fewness of the 20th-service litters, there seems to be a presumption in support of the idea, that by the time a male rabbit has performed twenty copulations within the space of three or four hours he is less able to beget large litters than when he has performed fewer than fifteen services within the same space of time.

When we consider in relation to each other the facts shown by tables 6 and 7 we are confronted by something of a puzzle. We have interpreted the facts shown in table 6, in part at least, as a direct consequence of the facts shown in the previous tables, i.e., that when the volume of semen, the number of sperm per cubic millimeter, the amount of progressive activity and the potential duration of motion of the sperm are all reduced, then the likelihood of spermatozoa reaching and penetrating the ova is reduced, and therefore the per cent of pregnancies is diminished. Table 6 shows a fairly regular and consistent decrease in certainty of pregnancy as sexual service increases. Now, as mentioned above, those circumstances which decrease the like-

likelihood of pregnancies should a priori reduce in almost like degree the size of those litters which are produced. But table 7 does not by any means indicate such a regular and consistent decrease in size of litter. In fact, up to the 15th service there is no perceptible falling off in litter size whatever. It is possible of course to form hypotheses to account for this condition of affairs, but at this time we know of no sound basis on which this discrepancy between tables 6 and 7 may be satisfactorily dealt with.

There exists considerable discussion, but so far as the writers know, no carefully controlled experimentation to the effect that in multiparous animals the male is without influence on litter size; the millions of sperm supplied by any 'normal' male at a single ejaculation will, it is said, be more than ample to impregnate the ova liberated. Our own work indicates that for rabbits there is at least one condition, i.e., performing twenty services in a short time, under which 'normal' males may be unable to bring about complete development of the full quota of ova liberated by the female. It is not inconceivable that the reproductive system of supposedly and apparently 'normal' males may be chronically in a condition analogous to that brought about in this experiment by excessive sexual service.

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EXPERIMENTS ON THE DEVELOPMENT OF THE SHOULDER GIRDLE AND THE ANTERIOR LIMB OF AMBLYSTOMA PUNCTATUM

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THIRTY-THREE FIGURES

1. INTRODUCTION

Experiments on the transplantation of limbs have been carried on in connection with various problems: a) on the development of nerves (Banchi, '05; Braus, '05, and Harrison, '07); b) on the question of orientation and laterality (Harrison, '17); c) on the question of the development of the shoulder girdle (Braus, '09), the rudiment of which is intimately bound up with that of the limb. It is with these last experiments that we are particularly concerned, since they have a more specific bearing on the results of the investigations set forth in this paper.

Although the intimacy of these two systems—shoulder girdle and limb—during development and differentiation led Wiedersheim ('92) to conclude that girdle formation is possible only under the formative influence of the free extremity, the experiments of Braus showed, in part, the lack of interdependence of these systems. Braus found that the removal of the fore-limb buds of *Bombinator* included the tissue from which the central or glenoid portion of the girdle develops, and that only the distal parts—suprascapula and epicoracoid—were formed following such an operation. The differentiation of these isolated girdle elements from unremoved blastema in the absence of the developing appendage demonstrated their independence of the free extremity. Confirmation of this observation was made on *Amblystoma* by Harrison ('18), who found, however, that as development proceeded these two separate distal elements gradu-

ally approximated each other until, in a larva which was kept alive eighty-five days after operation, they had become united into a single cartilage.

The formation of the suprascapula and coracoid in the absence of the glenoid portion of the girdle demonstrates that their rudiments are already determined at the time of the operation, and that, while they eventually grow together, their unremoved rudiments are nevertheless not capable of restoring the missing parts, viz., the scapula, all or only a portion of the procoracoid, and the shoulder joint, the rudiments of which are removed in a typical limb-bud extirpation.

Braus ('09) further found that when a limb bud is transplanted to a heterotopic position, a *complete* shoulder girdle of one-third to two-thirds the size of the normal develops at the place of implantation. From this he concluded (page 271) that the shoulder-girdle rudiment constitutes an equipotential restitution system.

According to this conclusion, totipotency is restricted to those girdle-forming cells which become implanted along with the limb bud, for, as has already been pointed out, the unremoved blastema can develop only into those parts the rudiments of which are already determined at the time of operation. The formation of a reduced girdle, with all its components, from cells which, in the normal environment, give rise to only the more central parts, would show that in their normal surroundings their prospective potency is greater than their prospective significance.

The results of the experiments set forth in this paper seem to necessitate for *Amblystoma*, however, an interpretation different from that which Braus placed on the results of his experiments.

This investigation was taken up at the suggestion of Prof. R. G. Harrison. It gives me pleasure to express here my thanks to Dr. Harrison for the guidance he has given me during its completion.

2. NORMAL ANATOMY

In order that the experiments may be more fully understood, a description of the normal girdle will first be given. Chondrification of the girdle is practically complete in a larva about twenty days after the closure of the medullary folds. The girdle then consists of a cartilaginous structure lying within the body wall and extending from the lateral aspect of the third myotome almost to the mid-ventral line (fig. 6). It is made up of the following components: a) the suprascapula, which consists of a rod-shaped element lying external to the pronephros and constituting the greater portion of the dorsal zone (fig. 23, *s.sc*); b) the scapula, which lies just dorsal and anterior to the glenoid cavity and which, in the cartilaginous state, is continuous with the suprascapula (fig. 23, *sc*); c) the procoracoid lying immediately anterior and slightly ventral to the glenoid cavity (fig. 23, *p.cor*), and d) the coracoid, a relatively broad expanse of cartilage, constituting nearly the entire ventral zone of the girdle and reaching close to the mid-ventral line (fig. 7, *cor.* and fig. 23, *cor.*). The scapula, procoracoid, and coracoid are continuous proximally and enter into the formation of the glenoid cavity which receives the head of the humerus (fig. 23, *gc*).

Chondrification

Chondrification of the girdle proceeds gradually from the central portion towards the periphery. There are three centers, one for the scapula, one for the coracoid, and one for the procoracoid. The center for the scapula is first to appear. This is followed by the center for the coracoid and finally by the procoracoid center. This was found by Wiedersheim ('89) to be the case with Triton, Siredon, and Salamandra. The same observation was also made by Braus ('09) on Bombinator.

The union of these three centers completes the chondrification of the central portion of the girdle. The suprascapula has no separate center and chondrification of this element proceeds gradually from the region of the scapula in a dorsal direction.

While the central part of the girdle is chondrified before the first two digits of the fore limb are fully formed and before the elbow joint becomes visible, the dorsal portion of the suprascapula is not entirely chondrified until the fourth digit makes its appearance. The chondrification of the coracoid likewise proceeds gradually from its center towards the mid-ventral line.

These observations agree with those of Braus ('09) on *Bombinator*. In this form there are no chondrification centers for the suprascapula and the epicoracoid. The epicoracoid of *Bombinator* is homologous with the ventral portion of the coracoid in *Amblystoma*, which, as has been pointed out, chondrifies gradually from the proximal part towards the periphery.

The cartilage center for the humerus appears somewhat earlier than do those for the girdle. Considering the *Amphibia* as a whole, it can be said that in most cases this is true (Wiedersheim, '89, '90, '92; Lignitz, '97, and Braus, '09). From Strasser's ('79) description of *Triton*, one would assume, however, that in this form initial chondrification of the humerus and the girdle takes place simultaneously.

The centers for the ulna and radius appear slightly later than those for the girdle, but they are completely chondrified before chondrification of the suprascapula and the coracoid have been completed.

The greater part of the girdle remains cartilaginous throughout life, but the entire scapula and those portions of the procoracoid and coracoid which enter into the formation of the glenoid cavity become ossified. The cartilaginous suprascapula which, in the larva, is a long slender rod-shaped structure, elongates in an antero-posterior direction so as to become a broad flat plate. The procoracoid grows out in an antero-ventral direction and becomes a structure very similar in shape to the procoracoid of *Necturus*. The coracoid, which comprises the greater part of the ventral zone of the girdle, is a large flat rounded plate of cartilage lying ventral and posterior to the procoracoid. The two coracoids overlap in the mid-ventral line. The shape of the ventral portion of the adult girdle is very similar to that figured by Fürbringer ('73) for *Salamandra maculata*.

No description of the shoulder muscles of *Amblystoma* could be found in the literature. The musculature, however, so far as has been studied, closely resembles that of *Salamandra maculata*, a European tailed Amphibian described by Fürbringer (op. cit.). In referring to the musculature, Fürbringer's nomenclature will be employed.

3. EXPERIMENTAL

The experiments were carried out upon embryos in two different stages: a) the so-called tail-bud stage, and b) the stage of open medullary folds.

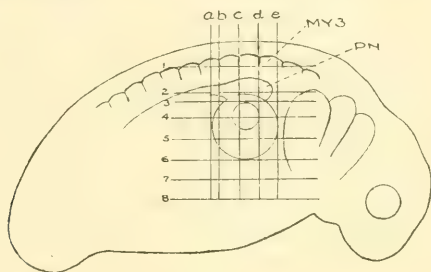


Fig. 1 Camera-lucida drawing of an embryo of *Amblystoma* in the tail-bud stage. The larger of the two circles represents the typical limb disc. $\times 15$. *pn*, = pronephros.

1. EXPERIMENTS ON EMBRYOS IN THE TAIL-BUD STAGE (STAGE 29)

A. Extirpation experiments

1. *Removal of the suprascapula rudiment.* As has already been pointed out (Harrison, '15), the fore-limb rudiment of an embryo in the tail-bud stage consists of a somatopleural thickening just ventral to the pronephros, centering in the region of the fourth myotome and extending over into that of the third and fifth. The formation of a suprascapula following the extirpation of the limb rudiment in this stage shows that its rudiment is not included with the limb mesoderm. Although there is no visible suprascapula rudiment, nevertheless, extirpation of the region *a-e* $\times 1-3$ (text fig. 1), including the outer or cutis layer of the

TABLE 1

Showing the results following the removal of the area $a-e \times 1-3$ (text fig. 1) including the outer cell layer of the somites and the pronephros

| INDIVIDUAL | AGE DAYS AFTER OPERA- TION | CONDITION OF THE GIRDLE AND THE EXTREMITY | | | | |
|------------|--|---|---------|-------------|----------|---------|
| | | Suprascapula | Scapula | Procoracoid | Coracoid | Humerus |
| E 8..... | 26 | absent | present | present | present | present |
| Ba 10.... | 24 | absent* | present | present | present | present |
| R 4..... | 26 | absent | present | present | present | present |
| E 6..... | 26 | absent | present | present | present | present |

* Only dorsal half wanting.

ventral halves of the somites and the pronephros, results in the formation of a girdle without a suprascapula (fig. 24 and table 1). The removal of this area has practically no effect on the development of the extremity itself and of the remainder of the girdle. Abnormalities in the limb sometimes occur if its rudiment is disturbed to any great extent during the operation.

2. *Removal of the suprascapula rudiment and of the myotomes.* This type of experiment consisted in the entire removal of the third, fourth, and fifth somites and the pronephros. In these experiments the wound was usually covered with ectoderm taken from a second embryo, since complete exposure of the notochord and the medullary tube frequently results in a disintegration of the embryo. Removal of the entire somites produces the same effect on the girdle as that described for the first type of experiment, viz., the formation of a girdle without a suprascapula (figs. 8 and 25 and table 2). In the absence of the somites, the limb and the girdle undergo a dorsal shifting (fig. 8). This is apparently due to a release of pressure from that direction.

Complete removal of the third, fourth, and fifth somites, while leading to no defects in the limb musculature, brings about marked deficiencies in the ventro-lateral musculature, observations corroborating those previously made by Miss Byrnes ('98) and Lewis ('10). Miss Byrnes (op. cit.) produced the first experimental evidence to show that the musculature of the anterior limb of *Amblystoma* develops in situ and that it is in no way derived from the myotomes or their ventral processes.

TABLE 2

Showing the results following complete removal of the third, fourth, and fifth somites and the pronephros

| INDIVIDUAL | AGE DAYS AFTER OPERATION | CONDITION OF THE GIRDLE AND THE EXTREMITY | | | | |
|------------|-----------------------------------|---|---------|-------------|----------|------------------|
| | | Suprascapula | Scapula | Procoracoid | Coracoid | Humerus |
| Ba 13.... | 23 | absent | present | present | present | present |
| Ba 11.... | 23 | absent | present | present | present | undifferentiated |
| Ex 1..... | 26 | absent | present | present | present | absent |
| E 7..... | 27 | dorsal half absent | present | present | present | present |

The absence of the limb in case Ex 1 is no doubt due to the destruction of its rudiment in the removal of the pronephros.

Lewis (op. cit.), however, in addition to demonstrating this same fact, showed that definite defects in the ventro-lateral musculature follow the removal of the myotomes of the limb region.

An examination of figure 8 will show that, in this case, there is complete absence of the ventro-lateral musculature on the operated side. This is, however, not always the case, for in others in which these same myotomes were completely excised, the ventro-lateral musculature is partly filled in by a compensatory elongation of the derivatives of intact myotomes. This same observation was also made by Lewis.

In the absence of the suprascapula, the m. dorsalis scapulae, which normally runs from the proximal end of the humerus over the external surface of the suprascapula (fig. 7, *m.dsc*), now attaches to the scapula (fig. 8).

It is essential, in order to remove successfully the suprascapula rudiment to include the pronephros. Its removal facilitates cleaning of the wound and thus affects indirectly the results of the experiment even though it exerts no direct influence. This applies as well to removal of the limb bud as a whole (Harrison, '15).

Since removal of the outer portions of the third, fourth, and fifth somites suppresses development of the suprascapula and since there is no visible rudiment for this element at the time of the operation, the experiments show that the suprascapula is

derived from tissue other than that which gives rise to the limb and the remainder of the girdle. Sections of older embryos show that dorsal to the pronephros scattered mesenchyme cells gradually appear and become continuous, external to the pronephros, with the limb-forming cells. It is evident that this mesenchyme, which later forms the suprascapula, is segregated from the outer or cutis layer of the somites in this region and that the suprascapula is formed in situ. Such a conclusion is strengthened by the fact that after complete removal of the limb rudiment and the pronephros, the suprascapula develops in its normal place, provided the third, fourth, and fifth somites are undisturbed.

Not only do these experiments show that the suprascapula is already determined at the time of the operation and that it is formed in situ, but they demonstrate as well the impotency of the unremoved girdle tissue to replace the missing part.

3. *Removal of the dorsal zone rudiment of the girdle and the limb mesoderm.* This series of experiments consisted of the removal of the area a-e \times 1-5 (text fig. 1). This included the outer portion of the ventral halves of the somites, the pronephros, and the limb mesoderm. The wounds were cleaned and covered. The removal of this area suppresses development of the suprascapula, scapula, and the free extremity, and only the ventral half of the girdle develops, no glenoid cavity being formed in any of these cases (figs. 9 and 26 and table 3). The formation in situ of the procoracoid and coracoid is evidence that they, too, are already determined at the time of the operation and are not dependent for differentiation on the remainder of the girdle and the limb, the rudiments of which were removed in this type of experiment. Although the myotomes proper were left intact, several larvae showed slight defects in the ventro-lateral musculature. This is no doubt due to a partial injury of the ventral portions which furnish the muscle buds.

The rudiments of practically all the shoulder musculature are included in these extirpations. In one case a few partially developed muscle fibers were present just external to the unremoved girdle elements. They probably represent the m. supra-

TABLE 3

Showing the results following the removal of the area $a-e \times 1-5$ (text fig. 1) including the outer portion of the somites, the pronephros, and the limb mesoderm

| INDIVIDUAL | AGE | CONDITION OF THE GIRDLE AND THE LIMB | | | | |
|------------|-------------|--------------------------------------|---------|-------------|----------|---------|
| | | Suprascapula | Scapula | Procoracoid | Coracoid | Humerus |
| | <i>days</i> | | | | | |
| R 6..... | 26 | absent | absent | present | present | absent |
| E 2..... | 26 | absent | absent | present | present | absent |
| R 2..... | 26 | dorsal portion present | absent | present | present | absent |
| E 10..... | 28 | absent | absent | present | present | absent |
| Ex 3..... | 28 | absent | absent | present | present | absent |
| R 1..... | 26 | small nodule of cartilage | absent | present | present | absent |

Incomplete absence of the suprascapula in cases R 1 and R 2 is apparently due to imperfect removal of the rudiment.

coracoideus, a muscle which normally runs from the proximal end of the humerus to the external surface of the coracoid (fig. 7, *m.spc*).

4. *Limb-bud extirpations.* The effects of the removal of a typical limb disc on the girdle (text fig. 1) are in accord with those described by Harrison ('18). In individual H 2 sectioned twenty-two days after the operation, only the suprascapula, a very small procoracoid, and the coracoid were present (fig. 10 and table 4). In another, H 5, only the dorsal part of the suprascapula was present in addition to a fragmentary procoracoid and the coracoid (fig. 27 and table 4). In these cases the pronephros was removed with the limb bud, and the incompleteness of the suprascapula in the second case is no doubt due to a partial destruction of its rudiment in the removal of the pronephros.

Since no limbs developed in experiment 3 after the removal of the area $a-e \times 1-5$, it is obvious that the ventral portion of a typical limb disc (Harrison, '15 and '18) which is shown in text figure 1 contains only girdle-forming cells.

The formation of only a portion of the ventral zone of the

TABLE 4

Showing the effects on the girdle of removal of the limb disc

| INDIVIDUAL | AGE | CONDITION OF THE GIRDLE | | | | |
|------------|-------------|-------------------------|---------|-------------|----------|----------------|
| | | Suprascapula | Scapula | Procoracoid | Coracoid | Shoulder joint |
| | <i>days</i> | | | | | |
| H 2..... | 20 | present | absent | present | present | absent |
| H 5..... | 20 | present* | absent | fragmentary | present | absent |

* Dorsal half only.

girdle following a typical limb-bud extirpation not only indicates that part of its rudiment is removed with the limb bud, but that the part which is unremoved is already determined at the time of the operation. Further, when a limb bud is transplanted to a heterotopic position, the development of a girdle with a ventral zone of reduced size (fig. 28) also serves to indicate that only a portion of the rudiment is transferred with the limb cells.

If localization of the cells which are to form the ventral zone of the girdle is complete at the time of the operation and they can be successfully extirpated, then only the dorsal zone should develop following their excision. A series of experiments were therefore performed to test the validity of this hypothesis.

5. *Removal of the rudiment of the ventral zone of the girdle.* It is obvious, in the light of experiments previously described (A 3 and A 4), that, in attempts to extirpate the entire rudiment of the ventral zone, the excision must include not only a portion of the limb bud, but tissue lying still more ventral than this area. In these experiments the mesoderm within the area a-e \times 4-8 was excised and the wounds cleaned and covered. While in seventy-five per cent of the cases normal limbs developed following excision of this area, it was found that, in a number of larvae sectioned twenty days after the operation, the ventral zone of the girdle was entirely wanting while the dorsal zone had developed normally (figs. 11, 12, and 29 and table 5). Several cases similar to the one figured, in which the entire rudiment of the ventral zone had been removed, not only lacked the procoracoid and coracoid elements, but the shoulder joint as well.

TABLE 5

Showing the results following the removal of the mesoderm within the area a-c \times 4-8 (text fig. 1)

| INDIVIDUAL | AGE | CONDITION OF THE GIRDLE AND THE EXTREMITY | | | | |
|-----------------|-------------|---|---------|-------------|-------------|---------|
| | | Supra-scapula | Scapula | Procoracoid | Coracoid | Humerus |
| | <i>days</i> | | | | | |
| Ext. Gr. C. 4. | 20 | present | present | absent | rudimentary | present |
| Ext. Gr. C. 9. | 20 | present | present | absent | rudimentary | present |
| Ext. Gr. C. 13. | 20 | present | present | absent | absent | present |
| Ext. Gr. C. 19. | 20 | present | present | absent | absent | present |
| Ext. Gr. C. 20. | 20 | present | present | absent | absent | present |

The development of a rudimentary coracoid in cases 4 and 9 is no doubt due to incomplete removal of the ventral zone rudiment.

The dorsal zone in these cases ended blindly at the point where the humerus began and was ankylosed with it. Shoulder movements in most of the cases were very slight or entirely wanting. In complete absence of the ventral zone, the procoraco-humeral and supracoracoideus muscles were also lacking, their rudiments having been removed with those of the cartilaginous elements. The pectoral muscles, however, were present and followed their normal course.

In other cases following the extirpation of this region the function of the limbs was more normal. It was found when these larvae were sectioned that, in addition to an imperfectly formed glenoid cavity, a small process of cartilage extended ventral to the proximal end of the humerus which could be identified as a very rudimentary coracoid. A short supracoracoideus muscle connected this fragmentary coracoid with the humerus. No procoracoid element could be distinguished.

The development of the rudimentary coracoid in these cases is evidence of incomplete removal of the entire rudiment of the ventral zone. Such cases, while they must be regarded in one sense as unsuccessful, have nevertheless an important bearing on the question of totipotency of the girdle-forming tissue. If the unremoved cells which formed this rudimentary coracoid were equipotential, then restitution of the entire ventral zone would be expected. This not being the case, it must be said that what

TABLE 6

Showing the results following removal of the mesoderm within the area a-e \times 3-8 (text fig. 1)

| INDIVIDUAL | AGE | CONDITION OF THE GIRDLE AND THE EXTREMITY | | | | |
|---------------------|-------------|---|---------|-------------|----------|---------|
| | | Supra-scapula | Scapula | Procoracoid | Coracoid | Humerus |
| | <i>days</i> | | | | | |
| Ext. Gr. C. 20..... | 20 | present | absent | absent | absent | absent |
| Ext. Gr. C. 25..... | 20 | present | absent | absent | absent | absent |

has been formed is an expression of the potency of these cells to form only that for which they are determined at the time of the operation. The rudimentary coracoid in these cases is smaller than that which developed from the tissue carried along with the limb cells in a typical limb-bud transplantation, but more coracoid-forming tissue is present in the latter case.

It is seen by referring to figure 1 that in the extirpation of the area a-e \times 4-8 slightly more than the ventral half of the typical limb disc is included, yet fifteen out of twenty such cases developed normal limbs. This high percentage of normal limbs from only the more dorsal portion of the rudiment affords additional evidence of the true equipotentiality of the limb system.

6. *Removal of the ventral zone rudiment of the girdle and the entire limb rudiment.* This type of experiment consisted in the removal of the mesoderm within the area a-e \times 3-8 (text fig. 1). All the wounds were cleaned and covered. The part removed included not only the limb mesoderm, but the rudiments of all parts of the girdle except the suprascapula. The results of these experiments are shown in figures 13 and 30 and in table 6.

The development in these cases of the suprascapula in the absence of the limb and all other parts of the girdle lends additional evidence of the independence of this element in its development, and shows further that it develops from a separate rudiment (Exp. A1, A2, and tables 1 and 2).

Braus was not able to say definitely whether or not in Bombinator the suprascapula had a separate rudiment. He was able to recognize two thickenings in the dorsal zone (Braus, '09, p. 175, fig. 5, *scap.*, and fig. 7, Z) which, however, were not

sharply marked off from each other, thus suggesting the possibility of their being a single anlage.

While more or less definite defects in the girdle have resulted from the extirpation of regional areas hitherto designated, the girdle defects following the removal of either the anterior or posterior half of the limb disc have not been so definite nor constant in character. The few specimens used in this study were given over by Dr. Harrison, who removed from the embryos the anterior and posterior halves of the limb disc in connection with his experimental study of the equipotentiality of the limb.

Three cases were studied in which the anterior half of the disc had been removed. In cases Rem. E. 22 and 23, preserved forty-six and eighteen days, respectively, after the operation and in each of which the limb had developed normally, the only defect in the girdle consisted in the absence of the procoracoid. In case H. R. E. 11—(twenty-six days) in which the limb consisted of nothing more than a nodule of undifferentiated mesenchyme cells, there was complete absence of the girdle. This case bears no significance with regard to localization, since ordinarily after the removal of the *entire* limb disc, the suprascapula, procoracoid, and the coracoid develop (Exp. A 4 and table 4). The only point of interest that this case offered was the fact that in the absence of skeletal differentiation, two of the shoulder muscles were differentiated, these being the trapezius and the pectoral.

Three cases were studied in which the posterior half of the limb disc had been removed. Case Rem. E. 12 (twenty-six days) with normally developed extremity showed considerable defects in the ventral zone of the girdle. The procoracoid was entirely wanting, while the coracoid was greatly reduced in size. The dorsal zone was normal. In case H. R. E. 12—(twenty-six days) with defective hand, there was no procoracoid. The coracoid, while being normal in length, was somewhat reduced in width. The dorsal zone was also poorly developed. In H. R. E. 9—(twenty-six days) in which no limb had developed, all parts of the girdle were present although somewhat atypical in shape. The most pronounced abnormality consisted of a distinct exchondrosis from the posterior border of the suprascapula.

Reviewing these few cases as a whole, we see that they showed no *consistent* defects, yet it is hardly to be expected that the removal of either the anterior or the posterior half of the limb disc would bring about any marked deficiencies in the girdle, since only a very small portion of the girdle rudiment can be involved in either case. Even after the removal of the entire limb disc, the suprascapula, procoracoid, and the coracoid develop in their normal position, while only the scapula and the glenoid portion of the girdle are wanting. It is quite possible that the excision of larger anterior or posterior areas would produce more pronounced and definite defects.

B. Transplantation experiments

The development of a limb in the absence of either the dorsal or the ventral half of the girdle suggested the possibility of its development in the absence of the entire girdle. Experiments were therefore made to test this possibility.

1. *Transplantation of small areas of cells from the dorsal half of the limb disc.* The area of mesoderm transplanted in these experiments is shown in text figure 1 by the smaller of the two circles. In five cases out of fifteen such transplants the tissue was resorbed. The other ten developed into limbs which showed more or less abnormality, consisting chiefly of reduplicated hands. Most of the limbs were atrophic and all lacked function. It was found when these larvae were sectioned twenty days after the operation that only a very fragmentary piece of the girdle was present, which from its relation to the humerus must be regarded as a rudimentary coracoid (fig. 14, *cor.* and fig. 31, *cor.*). This was connected with the humerus by a very short supracoracoideus muscle.

The almost complete absence of any girdle in these cases demonstrates that the region from which the transplanted tissue was taken is relatively free from cells which have the potency to form a girdle. When a larger area is transplanted, such as is indicated by the larger of the two circles in text figure 1, a con-

siderable portion of the girdle-forming cells is included so that a girdle of the size shown in figure 28 develops at the place of implantation.

2. *Transplantation of the ventral zone rudiment of the girdle and the ventral portion of the limb mesoderm.* It has been shown in table 5 that extirpation of the mesoderm within the area $a-e \times 4-8$ resulted in the formation of a girdle without a ventral zone (fig. 29). In this excision the ventral portion of the limb rudiment was included. This same area was transplanted in order to ascertain whether it would develop into a limb and a girdle consisting of only the ventral zone. Fifteen such transplantations were made. All developed limbs, of which ten showed reduplications of the hand; four were abortive and one normal. Three such cases were sectioned twenty-two days after the operation. The sections showed that only the ventral zone of the girdle was present with the limb (figs. 15 and 32). The limbs which resulted from these transplants developed from only a portion of the limb rudiment, showing thereby the equipotentiality of this system. The failure of the dorsal zone of the girdle to develop offers additional evidence to show the non-equipotentiality of the girdle rudiment.

3. *Transplantation of the dorsal zone rudiment of the girdle and the dorsal portion of the limb mesoderm.* This series of experiments consisted of the transplantation of the area $a-e \times 1-4$ (text fig. 1) including the ventral halves of the somites, the pronephros, and the dorsal segment of the limb mesoderm. Ten such transplants gave rise to limbs, eight of which showed either reduplications of the hand alone or of both the forearm and hand. An examination of the girdles which had developed in these experiments shows that they are composed almost entirely of a dorsal zone (figs. 16 and 33), which, while somewhat smaller than the dorsal zone of a normal girdle, are nevertheless complete with respect to their elements, viz., suprascapula and scapula. The presence of a very small portion of the ventral zone in the girdle shown in figure 33 is no doubt due to the inclusion in the transplant of some of the cells which are to form the ventral zone, yet the failure of these cells to form a complete

ventral zone shows again their lack of equipotentiality for this structure.

Experiments thus far described were made upon embryos at about the time of the appearance of the tail bud when the limb tissue is in the form of a definite limb bud. The relation of the girdle rudiment to that of the limb has been shown in these experiments. They have further served as a means of showing in two different ways—extirpation and transplantation—that not only is the girdle rudiment already determined at this stage, but that the rudiments of the separate components have already become localized.

A further discussion of these experiments will be taken up after the experiments in part 2 have been considered.

2. LIMB EXPERIMENTS ON EMBRYOS IN THE STAGE OF HIGH MEDULLARY FOLDS (STAGE 18) BEARING UPON THE DEVELOPMENT OF THE SHOULDER GIRDLE

A. Extirpation experiments

While the limb rudiment is present as a definite somatopleural thickening in the embryos used for all experiments hitherto described, its definiteness is gradually lost as we study successively earlier stages. In the stage of open medullary folds, no somatopleural thickening is present and the axial and the lateral mesoderm have not yet been separated. Therefore at this stage the limb rudiment is present as a region of mesoderm without visible local characteristics. Embryos at this stage also lack the surface markings, viz., somites and pronephros, which indicate the position of the limb rudiment in the older stage and which are used as landmarks in experimentation. Consequently, it is impossible to make *exact* determinations of the area of limb-forming cells.

Location of the limb mesoderm in the absence of surface markings was determined by a fairly accurate method, already described by the author (Detwiler, '17), a brief description of which may be repeated here.

After removing the embryos from the capsule a circular cut

was made just posterior to an imaginary line passing dorso-ventrally midway through the embryo. The ectoderm and mesoderm were removed. The wound, after being cleaned from all free mesoderm cells, was then covered over by a circular piece of ectoderm, cut to fit the excision, taken from an embryo which had been properly stained in an aqueous solution of Nile blue sulphate. In about one-half hour this stained disc of ectoderm had healed in and its size and position was then indicated by a camera drawing (text fig. 2). The heavily stippled area represents



Fig. 2 Camera drawing of an embryo (E. Tr. Ext. 12) in the stage of high medullary folds. The heavily stippled area represents a circular piece of ectoderm taken from an embryo previously stained in a solution of Nile blue sulphate. This occupies the wound resulting from the extirpation of the limb mesoderm. $\times 10$.

Fig. 3 Camera drawing of the same embryo (E. Tr. Ext. 12) shown in figure 2 thirty hours later, showing the position of the inserted stained disc of ectoderm with reference to the somites. $\times 10$.

the circular piece of transplanted blue ectoderm, which is in marked contrast to the brownish-yellow ectoderm of the host.

The position of this inserted piece of ectoderm, when development had proceeded so that the somites had become visible, served to indicate whether or not the limb rudiment had been removed. If the stained disc eventually came to lie just ventral to the third, fourth, and fifth somites (text fig. 3), then it could be said with more or less certainty that the limb rudiments had been removed. When the extirpated area was taken from a more posterior position than that shown in text figure 2, the stained disc which covered the wound came to lie ventral to the fourth, fifth, and sixth or the fifth, sixth, and seventh somites. This indicated that only a portion of the limb rudiment had been removed and regeneration could be predicted.

The position of the stained disc is not always an absolute indicator of the region of the excised mesoderm, since it may be changed somewhat by ectodermal shifting. There is, however, very little shifting in the immediate limb region. The more pronounced ectodermal shifting, which does not usually occur until a somewhat later stage, takes place dorsal to the limb region and proceeds in a dorso-posterior direction; the general character and direction of the movement being similar to that observed in frog embryos by Harrison ('03) in his experiments on the development of the lateral line.

The migrating ectoderm, however, occasionally involves the dorsal portion of the stained area which then loses its circular shape and lengthens out dorso-posteriorly. Even though the stained disc is not involved in the ectodermal migration, it gradually becomes larger through continued cell division and the stain, now being distributed over a larger area, gradually loses its intensity.

It is impossible to excise equal areas in all cases and a certain degree of variation is to be expected. Measurements of the antero-posterior and dorso-ventral diameters of the inserted disc, after healing, were taken in many cases. These, of course, represent approximately the diameters of the excised piece. The size of this area was compared with that of a typical limb bud, which, in an embryo of the tail-bud stage has a diameter of three and one-half somites, an actual measurement of 0.93 mm. The diameters of the discs extirpated from the embryos in the neural fold stage varied between 0.66 and 0.93 mm.

Harrison ('15 and '18) showed in a series of extirpation experiments on embryos in the tail-bud stage that no regeneration occurred from wounds the width of three and one-half somites in diameter, provided they had been cleaned and covered, but when they were only three somites in diameter 33 per cent regeneration occurred. In most of the experiments on the neural-fold stage the average diameter of the excised areas was equal to the width of from three to three and one-half somites, or 0.73 to 0.93 mm. The results of the simple extirpations are given in table 7.

Although slight variations in the position from which these

TABLE 7

Showing the effect of removal of the limb mesoderm from embryos in the stage of high medullary folds

| SIZE OF WOUND, DIAMETER | WOUNDS COVERED | | WOUNDS NOT COVERED | |
|----------------------------|----------------|-----------------|--------------------|-----------------|
| | Regenerated | Not regenerated | Regenerated | Not regenerated |
| <i>mm.</i> | | | | |
| Not recorded | 10 | 23 | 7 | 14 |
| 0.66 | 6 | 0 | | |
| 0.73* | 6 | 2 | | |
| 0.80 | 1 | 3 | | |
| 0.86 | 0 | 3 | | |
| 0.93† | 0 | 5 | | |

* Equals the size of a three-somite wound.

† Equals the size of a three-and-one-half-somite wound.

excised areas were taken undoubtedly occur because of the absence of definite surface markings, nevertheless, the results of the table show that, as the diameter of the excised area approaches the diameter of a three-and-one-half somite wound, less regeneration occurs.

While the limb mesoderm can be successfully extirpated from embryos of this stage certain technical difficulties are met with in the procedure. Although healing of the tissue is rapid, the embryos frequently disintegrate a day or two after the operation. It was found that a smaller number disintegrated if they were kept at a temperature of about 15°C. for twenty-four hours after the operation and then brought back to room temperature.

Watch glasses which were used in these operations were coated either with wax or paraffin in order to prevent the embryos from sticking to the bottom. If this is not done, they frequently adhere to the glass and the ectoderm is torn off in attempts to liberate them. Embryos should remain in these coated dishes for from twelve to twenty-four hours after the operation. They can then be transferred with safety to ordinary watch glasses.

B. Transplantation experiments

In the transplantation of the limb mesoderm the method of staining with Nile blue sulphate was also employed in a number of cases. Of two embryos which were selected, one was stained in an aqueous solution of the dye until the desired color had been attained. In the other embryo a circular wound was prepared, posterior to the region of the limb mesoderm, for the reception of the transplant. The limb mesoderm with the overlying ectoderm was then excised from the stained embryo (position indicated in text fig. 2) and transferred to the prepared



FIG. 4 Camera drawing of an embryo in the stage of high medullary folds. The heavily stippled disc indicates the size and position of the limb mesoderm with the overlying ectoderm transplanted from an embryo previously stained in a solution of Nile blue sulphate. $\times 10$.

Fig. 5 Camera drawing of the same embryo shown in figure 4 two days later, showing the relation of the transplanted area to the normal limb region. $\times 10$.

wound (text fig. 4). It is seen from this figure that a transplant so placed occupies the flank region at the very posterior end of the embryo. When the embryo had developed so that the somites became visible, the position of the stained transplant with respect to the somites, as well as its relation to the normal limb region, could be ascertained (text fig. 5). Transplants which are placed at the position indicated in text figure 4 practically always come to lie ventral to the sixth, seventh, and eighth somites (text fig. 5), a position which is so near the normal limb region that conflicts frequently arise in the development of the two systems. The limbs which usually start separate development very often fuse together. In several cases only one limb developed from the two systems. In order to obviate these

TABLE 8

Showing the results of transplantation of the limb mesoderm from embryos in the stage of high medullary folds

| ORIENTATION | NOR- MAL LIMBS | ABOR- TIVE | REDU- PLICA- TIONS | FUSION WITH NOR- MAL LIMB | ABNOR- MAL SINGLE LIMBS | TRANS- PLANT RE- SORBED |
|---------------------------------|----------------------|---------------|--------------------------|---------------------------------------|----------------------------------|----------------------------------|
| Homopleural dorso-dorsal..... | 8 | 2 | 10 | 7 | 2 | 3 |
| Homopleural dorso-ventral..... | 2* | 1 | 2 | 2 | 1 | 0 |
| Heteropleural dorso-dorsal..... | 2† | 1 | 1 | 1 | 4 | 0 |

* Laterality reversed.

† Original laterality retained.

abnormalities, the transplants were later placed at the posterior end of older embryos. In such cases the transplant was of sufficient distance from the normal limb to allow independent development of the two systems.

The region in the stained embryo from which the transplant had been taken was in many cases covered with unstained ectoderm taken from a third embryo. This unstained insert now occupied the place from which the transplant had been taken and its position in the embryo, when the somites became visible, served to indicate whether the limb rudiment had been entirely or only in part transplanted.

The results of the transplantation experiments are given in table 8.

The posture of the normal limbs is in accord with the fundamental rules 1 and 2 underlying the determination of laterality (Harrison, '17). When the transplant is grafted on the same side from which it is taken (homopleural) and not inverted (dorso-dorsal), original laterality is retained (rule 1), but when inverted (dorso-ventral) a limb of opposite laterality results (rule 2). When transplanted to the side opposite to that from which it is taken (heteropleural) and not inverted, original laterality is retained (rule 1).

While the number of experiments is small, the results show that the fundamental asymmetry of the limb mesoderm is already determined in embryos with high medullary folds.

Just how early the limb rudiment is determined cannot be said

at this time. It was successfully transplanted in several cases from embryos with low medullary folds, but no experiments have been made upon still younger embryos.

The region of mesoderm extirpated from the embryos in the stage of high medullary folds (text fig. 2) not only included the limb cells, but, in many cases, the entire girdle rudiment, so that there was complete absence of the girdle on the operated side (fig. 17). This figure is a section taken from the same embryo (E. Tr. Ext. 12) which is shown in text figure 2. The size of the region extirpated in this case corresponds to that of a three-somite wound in an embryo of the tail-bud stage, an extirpation which, in the later stage, removes only the central portion of the girdle rudiment so that isolated pieces of the girdle develop from the unremoved tissue (fig. 10). It is seen by comparing the two conditions resulting from extirpations of the same size that in the embryo with high medullary folds the girdle rudiment occupies a smaller area than in an embryo of the tail-bud stage.

The excised areas in the earlier stage always includes the rudiment of the pronephros. In the absence of the pronephros on the operated side a compensatory hypertrophy of this structure on the intact side is always found (fig. 17). This is in accord with Miss Howland's ('16) observations. Hypertrophy appears to be greater when the rudiment is removed from these early embryos than when the partially developed structure is removed from older embryos.

While in a number of these larvae, without the appendage, the entire girdle was lacking, in others a 'girdle' was found to be present (fig. 18). Although such girdles are present as continuous cartilaginous bands, nevertheless the central portion lacks those characteristics which typify the normal structure. This portion probably represents a hyperplastic growth of those parts which developed from the unremoved girdle cells which were located near the periphery of the wound. The completeness of these girdles therefore cannot be regarded as a manifestation of true restitution processes, for the completeness is only one of quantity and not of quality.

Since the region of mesoderm shown in text figure 2, when extirpated, frequently includes the entire girdle rudiment, it is obvious that when transplanted all or nearly all of the rudiment is included in the transplant.

When such transplantations were made it was found that the girdles which had developed at the place of implantation were almost as large as the normal girdles (fig. 20). Figure 19 represents a section through the normal girdle of individual D 29 preserved twenty-four days after the operation. Figure 20 shows a section through the implanted girdle. When these two girdles are compared and measured it is found that the implanted girdle is about three-fourths as large as the normal one. In individual D 41 preserved twenty-seven days after the operation the implanted girdle was also found to be three-fourths as large as the normal. In this case, as well as others, the implanted mesoderm was placed so near to the normal limb region that there was partial fusion of the two girdles. In individual D 23 preserved twenty-four days after the operation, the implanted girdle was larger than in all other operated cases studied, it being four-fifths the size of the normal. The dorsal zone was typical in shape, although the ventral zone was broader and not quite as long as that of the normal. There was also partial fusion of the two girdles.

The fact that double limbs frequently arise from a single transplanted rudiment has already been shown (Braus, '04 b, and Harrison, '07). When double limbs develop from transplants which are placed near the normal limb rudiment, the anterior of the two frequently unites with the normal limb to form a fused double limb, while the posterior member of the reduplication remains single. This condition was met with in individual D 23. Although the anterior member had fused with the normal limb, its shoulder joint was formed on the implanted girdle. A separate shoulder joint was formed some distance posterior and ventral to this for the reception of the posterior member of the reduplication. Other cases have shown that there may be only one shoulder joint for both members of a double limb. The formation of double limbs therefore does not mean the formation

of a double girdle. In all cases studied a single girdle was formed. If the two members of a double limb are completely fused at the base there is only one shoulder joint; if they are separated at the base two joints may be formed on the same girdle.

According to Braus ('09, p. 283), when reduplicating limbs arise from a single transplanted rudiment of *Bombinator*, there may be a complete reduplication of the implanted girdle; or, in addition to the formation of one main girdle for the principal limb, there may be an accessory girdle for the supernumerary appendage (Braus, *op. cit.*, p. 284, and pl. 16, fig. 5 a).

It is evident from the cases cited (D 23, 29, 41) in which girdles from three-fourths to four-fifths the size of the normal had developed, that all or nearly all of the rudiment must have been included in the transplant. Their being slightly smaller than the normal is probably due to the fact that they are placed in an abnormal environment where there is less space for differentiation than in the normal situation.

Girdles which develop from typical limb-bud transplantations in embryos of the tail-bud stage (text fig. 1) are never as large when compared with the normal as those which have just been described. When the girdle shown in figure 21, which is a typical result of such a transplant, is compared with the normal girdle of the same larva (fig. 22), it is found to be only one-third as large. The difference in size of the implanted girdles in the two cases becomes explicable when it is seen that in one case (fig. 21) only the central portion of the girdle rudiment is transplanted with the limb mesoderm, while in the other (fig. 20) all, or nearly all, of the rudiment is included.

4. DISCUSSION

In the embryos of *Amblystoma* individual portions of the shoulder-girdle rudiment are determined at the stage when the limb tissue is present as a definite somatopleural thickening (stage 29). Experimental evidence has been advanced to show that after extirpation of any definite portions of the rudiment

only those components develop which are represented in unrecovered tissue. Simple limb-bud extirpations (Exp. A 4, p. 507) demonstrate localization of only the more distal portions of the rudiment, since in the absence of the central portion, which is removed along with the limb bud, isolated elements (suprascapula and coracoid) of the girdle are formed. However, the formation of only the dorsal zone following complete removal of the rudiment of the ventral zone (Exp. A 5, p. 508) or just the opposite experiment (Exp. A 3, p. 506) offers proof that the central portion of the rudiment is also localized and that the extirpation of any given portion does not incite restitution processes in the unrecovered parts.

The system at this stage may be regarded as a mosaic of which the various parts may develop into later known components without influence of neighboring portions. Braus ('09, p. 268) also called the shoulder-girdle system of *Bombinator* a mosaic, but only in its normal situation. According to him, when a portion of its rudiment is transplanted to a new environment, the mosaic is lost and the implanted tissue acquires the capacity of reversing differentiation which had already appeared in the anlage, so that it is now able to form anew an entire girdle. Accordingly, transplantation incites restitution, and the system, which in its normal environment is a mosaic, in the new environment becomes an equipotential one.

Although Braus found that the shoulder girdles which developed from implantations of only a portion of the rudiment were of reduced size (one-third to two-thirds the size of the normal), nevertheless, they were regarded by him as entire, just as complete individuals of reduced size are formed from isolated blastomeres in the cleavage stage of an egg or from fertilized egg fragments. While the girdles which had developed at the implanted place were formed in all cases from the central portion of the rudiment, since it is this portion which is included with the limb cells, Braus nevertheless questioned whether there was any greater inherent capacity on the part of any portion of the anlage over any other in the process of restitution. According to this idea, the entire structure might develop from

either the dorsal or ventral parts of the rudiments, yet no experiments were made to test the validity of this hypothesis.

While Braus ('09, p. 27) claimed that this system in *Bombinator* is equipotential from stage 2, when the limb bud is very small, up to the time of chondrification of the humerus, it is clear from experiments described in this paper that such is not the case in *Amblystoma*.

It has been shown, that when only the ventral portion of the rudiment was transplanted with the limb cells, only the corresponding zone of the girdle developed (figs. 15 and 32), just as when the dorsal half of the rudiment was transplanted with the limb cells, it developed into a girdle which was composed almost entirely of a dorsal zone (figs. 16 and 33). The presence of a very small portion of the ventral zone in these cases is no doubt due to the inclusion in the transplant of some of the cells which are to form the ventral zone, since it is difficult to separate accurately the two halves of the rudiment. The failure, however, of these cells to form a complete ventral zone shows their lack of the potency to form this portion of the girdle.

Although the girdle rudiment of *Amblystoma*, when transplanted, may not be regarded as a perfect mosaic, since slightly more cartilage may form from portions of the rudiment than would have formed had they not been transplanted, yet it is obvious that it cannot be regarded as an equipotential system at the stage with which we are dealing. In a true equipotential system such as the limb rudiment, the extirpation of any portion does not lead to any definite defects in the components of the limb which develop from the unremoved portion of the rudiment. Even after extirpation of a half of the rudiment, normal limbs with all their cartilaginous and muscular elements may develop. The same thing is true when a half of the limb rudiment is transplanted. In this case two limbs of normal size and structure may develop, one from the transplant and the other from the unremoved half of the rudiment.

Definite defects in the girdle resulting from removal of certain portions of its rudiment, as well as the formation of girdles with only dorsal or ventral halves resulting from the transplantation

of the corresponding portions of the rudiment, are sufficient evidence to show that the system cannot be regarded as being equipotential at the stage when the limb mesoderm is in the form of a definite limb bud.

Experiments upon embryos in the stage of open medullary folds show that the girdle rudiment occupied a smaller area than in embryos of the tail-bud stage and that it was frequently entirely removed along with the limb cells so that no girdle developed. In other cases where it was not entirely removed a complete cartilaginous band was formed which, upon superficial examination, resembled a normal girdle (fig. 18). This cannot be regarded necessarily as a complete girdle, however, for it is lacking in certain components which typify the normal structure. The central portion of a girdle of this type no doubt represents a hyperplastic growth of the more distal portions whose rudiments were not removed with the excised area, and the completeness of this structure must be regarded as one of quantity rather than one of quality. From the incompleteness of quality of a structure of this type, it is seen that even at the stage of open medullary folds the system is not capable of qualitative restitution. This is strengthened by the fact that in several cases the girdles which had developed from implanted mesoderm at this stage were composed of only the ventral zone, indicating that only the corresponding portion of the rudiment was transplanted.

It has been shown that girdles which develop from transplanted areas in stage 18 were considerably larger than those which formed from areas of equal size in stage 29 (compare figs. 20 and 21). This condition might be used as a point in favor of equipotentiality of the system in the early stage, were it not known that in the former case all or nearly all of the rudiment was transplanted, while in the latter case only the central portion.

Braus ('09, p. 277) claimed that, between his stage 2, when the limb bud first becomes visible, and the stage when chondrification of the humerus begins, an area of transplanted mesoderm taken from a younger embryo did not develop into a girdle any

larger than that which developed from the same sized area from an older embryo. As has already been shown, this is not the case in *Amblystoma* between the stage of open medullary folds and the tail-bud stage. These stages, however, are younger than any used by Braus.

Variations, therefore, in the size of the implanted girdle are to be regarded as an expression of a greater or less amount of girdle rudiment having been included with the transplanted limb mesoderm.

5. SUMMARY

1. The separate parts of the shoulder-girdle rudiment of *Amblystoma punctatum* are already determined at the stage when the limb rudiment is present as a definite thickening of the somatopleure (Stage 29).

2. The removal of a definite portion of the rudiment brings about a defect in that portion of the girdle corresponding to the part removed.

3. The extirpation of a given portion of the rudiment does not incite restitution processes in the unremoved parts. In the absence of the central part of the girdle the gap may be bridged over by a hyperplastic development of those elements which are formed from the unremoved portions of the rudiment.

4. The suprascapula develops 'in situ' from a separate rudiment, the extirpation of which has no effect on the development of the limb nor on the remainder of the girdle (Exp. A 1, fig. 24, and table 1).

5. Complete extirpation of the third, fourth, and fifth somites, while having no effect on the musculature of the limb, does, however, produce marked defects on the ventro-lateral musculature (Exp. A 2). These results corroborate those of Byrnes ('98) and Lewis ('10).

6. The central portion of the girdle rudiment is carried along in the transplantation of a typical limb bud (text fig. 1) and a girdle of about one-third the size of the normal develops at the place of implantation (fig. 21; cf. fig. 22).

7. It is possible to initiate the development of a limb in

almost complete absence of the shoulder girdle by transplanting a small area of cells (text fig. 1, smaller of the two circles) from the dorsal half of a typical limb disc (fig. 31). This area is practically free from girdle-forming cells.

8. Either the dorsal or ventral half of the girdle rudiment may be transplanted along with the corresponding portions of the limb rudiment. In such cases complete limbs develop from the transplanted portion of its rudiment, while only that portion of the girdle develops corresponding to the part of the rudiment which has been implanted (Exps. B 2, B 3, and figs. 32 and 33). These results demonstrate the non-equipotentiality of the girdle system.

9. The limb mesoderm is already determined in embryos in the stage of open medullary folds. The rudiment can be successfully extirpated and transplanted at this stage (tables 7 and 8).

10. In the absence of surface markings, the position of the limb mesoderm can be located quite accurately by covering the wound with ectoderm from an embryo, previously stained in a solution of Nile blue sulphate, and observing its relation to the later formed somites (text figs. 2 and 3).

11. When typical limb buds the width of three and one-half somites in diameter (text fig. 1) are extirpated from embryos in the tail-bud stage, only the central portion of the girdle rudiment is included (Exp. A 4, fig. 10); but areas of equal size when extirpated from embryos in the stage of high medullary folds (text fig. 2) frequently include the entire girdle rudiment (fig. 17).

12. Implanted girdles developing from areas of mesoderm, which are taken from embryos in the stage of open medullary folds, are considerably larger when compared with the normal than those which develop from areas of equal size taken from embryos in the tail-bud stage (cf. figs. 19, 20, 21 and 22).

13. When double limbs arise from a transplanted rudiment, only one girdle is formed, which may have one or two shoulder joints, depending on whether or not the two members of the reduplication are completely fused or partly separated at the base.

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PLATES

All of the figures in plates 1, 2, and 3 are microphotographs of transverse sections cut $10\ \mu$ in thickness and magnified 30 diameters. The larvae were fixed in sublimate-acetic killing fluid and the sections stained with Ehrlich's haematoxylin and Congo-red. All operations were made on the right side which, in the figures, appears as the *left*.

ABBREVIATIONS

| | |
|--|---|
| <i>cor</i> , coracoid | <i>p.cor</i> , procoracoid |
| <i>g.c</i> , glenoid cavity | <i>pn</i> , pronephros |
| <i>hum</i> , humerus | <i>sc</i> , scapula |
| <i>m.dsc</i> , musc. dorsalis scapulae | <i>s.sc</i> , suprascapula |
| <i>m.spc</i> , musc. supracoracoideus | <i>vlm</i> , ventro-lateral musculature |
| <i>my</i> , myotome | |

PLATE 1

EXPLANATION OF FIGURES

Fig. 6 A section through the shoulder girdle of a normal larva twenty-five days after the closure of the medullary folds.

Fig. 7 A section through the glenoid cavity and the coracoid portion of the girdle of a normal larva.

Fig. 8 A section through the shoulder region of individual Ba. 13, showing the effect on the girdle of the entire removal of the third, fourth, and fifth somites.

Fig. 9 A section through the shoulder girdle of individual E. 2 developed in the absence of the rudiments of the suprascapula, scapula, and the limb (a-e \times 1-5, text fig. 1).

Fig. 10 A section through the shoulder region of individual H. 2 showing the absence of the central part of the girdle, the rudiment of which is removed in the extirpation of a typical limb bud (text fig. 1).

Fig. 11 A section through the shoulder girdle of individual Ext. Gr. C. 19 which developed after the removal of the rudiments of the ventral zone (a-e \times 4-8, text fig. 1).

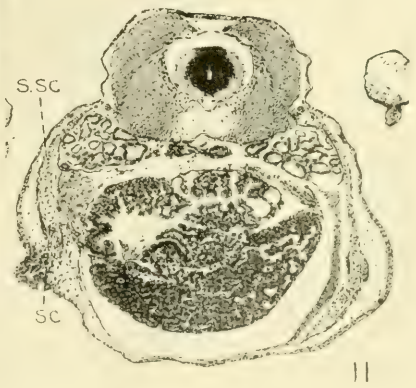
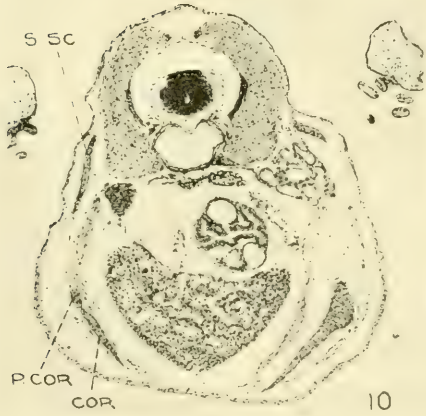
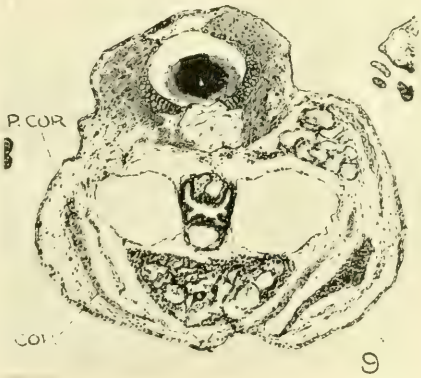
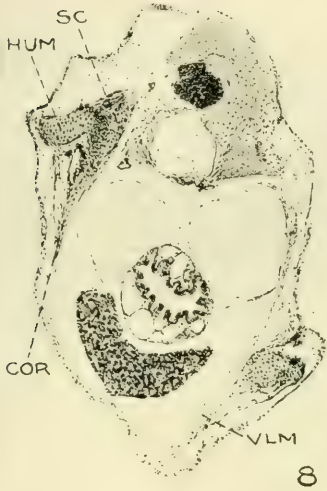
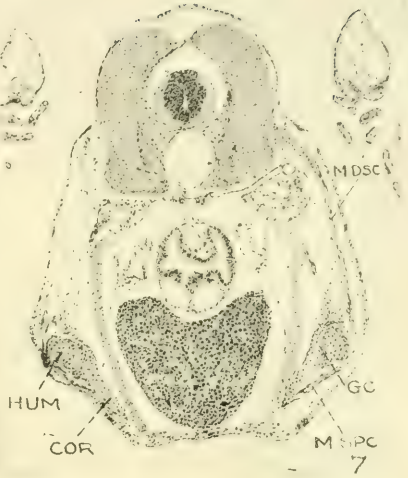
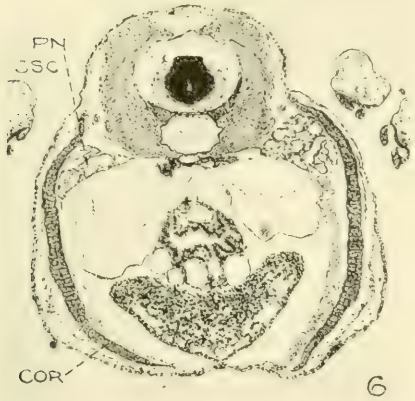


PLATE 2

EXPLANATION OF FIGURES

Fig. 12 A section through the shoulder region of individual Ext. Gr. C. 19 slightly posterior to that shown in figure 11, showing the absence of the glenoid cavity and the ventral zone of the girdle (compare with fig. 7).

Fig. 13 A section through the shoulder region of individual Ext. Gr. C. 25, showing the development of the suprascapula after the rudiments of all other parts of the girdle had been removed (a-e \times 3-8, text fig. 1).

Fig. 14 A section of individual S. B. 2, showing the presence of only a very fragmentary portion of the girdle which developed, along with the limb, from the transplantation of a small area of cells taken from the dorsal portion of the limb disc of another embryo (text fig. 1, smaller of the two circles).

Fig. 15 A section of individual Tr. 2/v 10, showing the girdle which developed in a heterotopic position from the transplantation, along with a portion of the limb mesoderm, of the ventral half of the girdle rudiment (a-e \times 4-8, text fig. 1).

Fig. 16 A section of individual Tr. d/2 4, showing the shoulder girdle which developed in a heterotopic position from the transplantation of the dorsal half of the girdle rudiment along with a corresponding portion of the limb mesoderm (a-e \times 1-4, text fig. 1).

Fig. 17 A section from the shoulder region of individual E. Tr. Ext. 12 showing complete absence of the girdle, the rudiment of which was removed along with the limb mesoderm from the embryo in the stage of open medullary folds (text fig. 2).

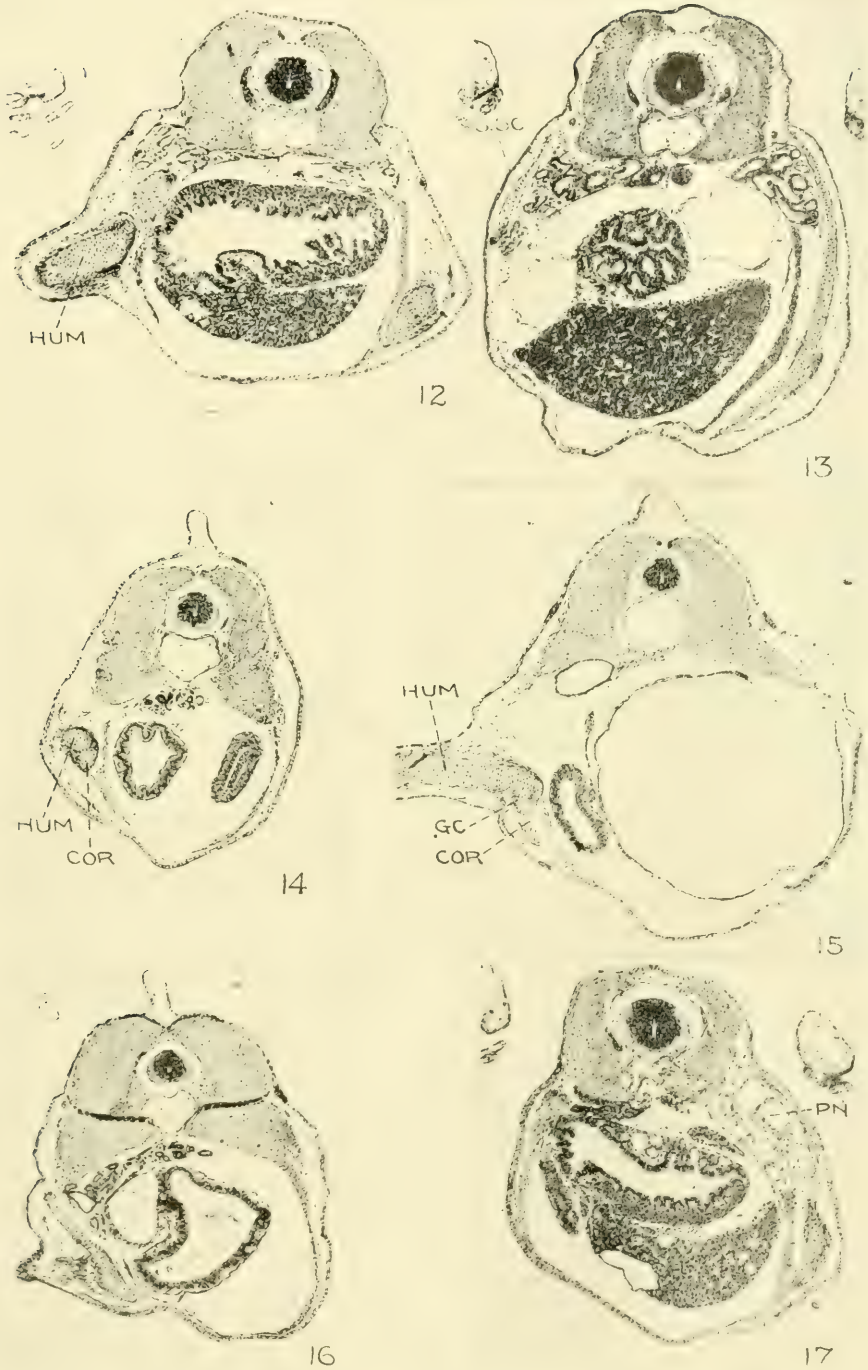


PLATE 3

EXPLANATION OF FIGURES

Fig. 18 A section through the girdle of individual Ca. 6 developed in the absence of the limb and the central portion of the girdle rudiment.

Fig. 19 A section through the normal girdle of individual D 29.

Fig. 20 A section from individual D 29 showing the girdle which developed in a heterotopic position from the implantation, along with the limb mesoderm, of the girdle rudiment from an embryo in the stage of open medullary folds.

Fig. 21 A section through the implanted girdle of individual Tr. Ext. 154 developed from the central portion of the rudiment which was transplanted, along with the limb rudiment from an embryo in the tail-bud stage.

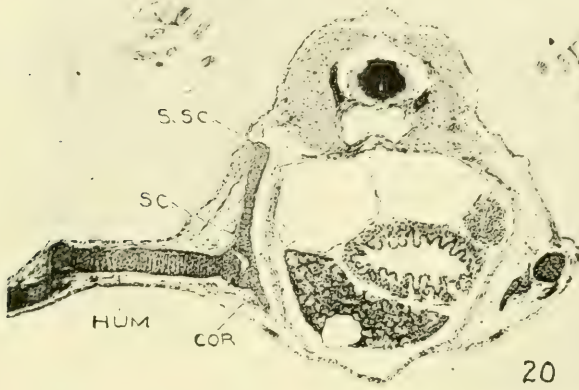
Fig. 22 A section through the normal shoulder girdle of individual Tr. Ext. 154.



18



19



20



21



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PLATE 4

EXPLANATION OF FIGURES

All of the figures are photographs of blotting paper reconstruction models of the shoulder girdle. The models were made at a magnification of 100 diameters and reduced one-half for publication.

Fig. 23 Model of the left normal shoulder girdle of individual Ba. 13.

Fig. 24 Model of the right shoulder girdle of individual R. 4 which developed in the absence of the suprascapula rudiment.

Fig. 25 Model of the right shoulder girdle of individual Ba. 13 developed in the absence of the third, fourth, and fifth somites.

Fig. 26 Model of the right shoulder girdle of individual E. 2 developed in the absence of the rudiments of the suprascapula, scapula and the limb (compare with fig. 9).

Fig. 27 Model of the right shoulder girdle of individual H. 5 developed in the absence of the central portion of the rudiment which was removed along with the limb bud (compare with fig. 10).

Fig. 28 Model of the implanted girdle of individual Tr. Ext. 154 developed from the central portion of the rudiment which was transplanted along with a typical limb disc (compare with fig. 21).

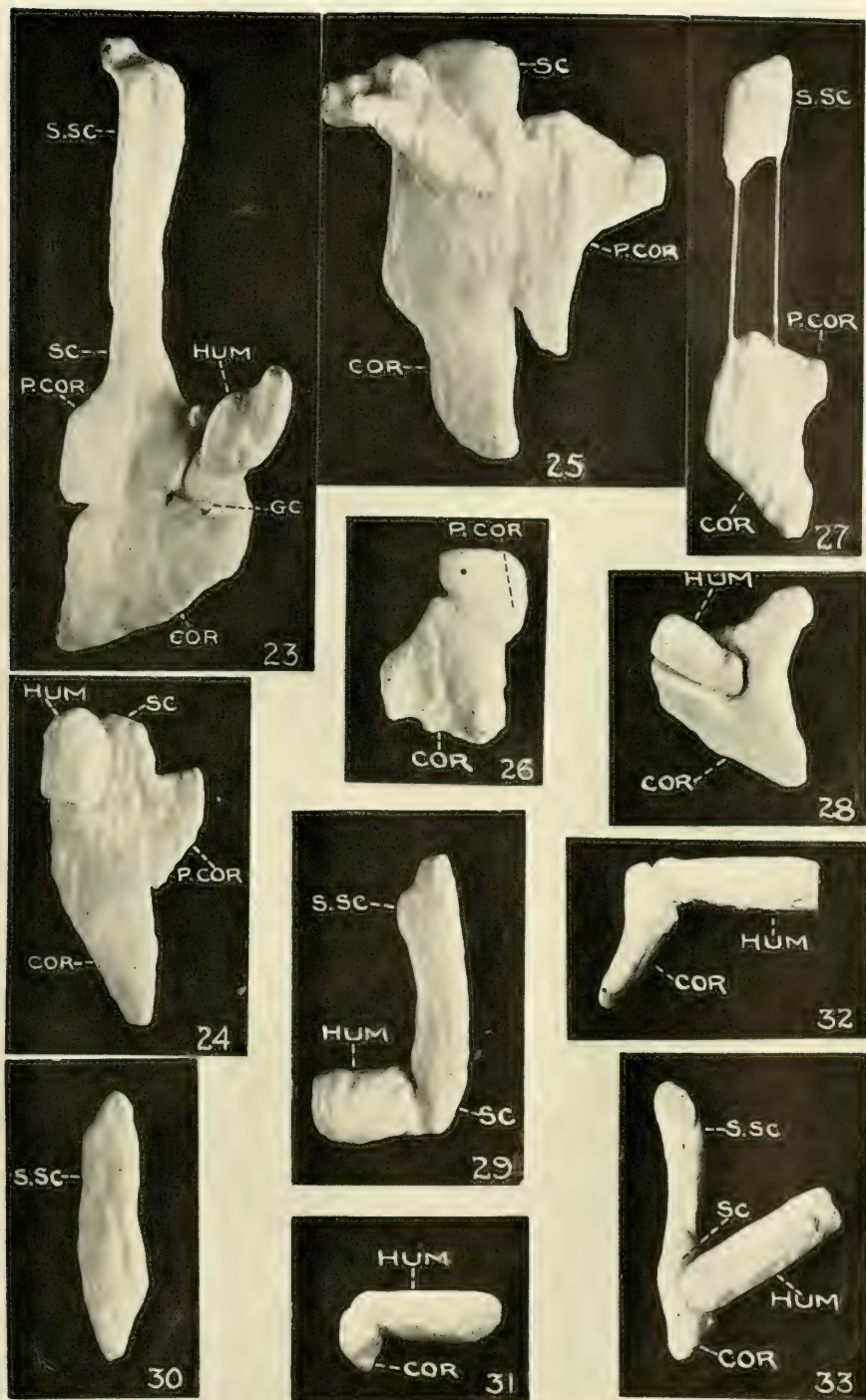
Fig. 29 Model of the right shoulder girdle of individual Ext. Gr. C. 13 developed in the absence of the rudiment of the ventral zone (compare with figs. 11 and 12).

Fig. 30 Model of the right suprascapula of individual Ext. Gr. C. 25 developed in the absence of the limb and the remainder of the girdle (compare with fig. 13).

Fig. 31 Model showing a very fragmentary girdle which developed, along with the limb, from the implantation of a small area of cells taken from the dorsal portion of the limb disc (compare with fig. 14).

Fig. 32 Model of an implanted girdle from individual Tr. 2/v 10 which developed in a heterotopic position from the transplantation of the ventral zone of the girdle rudiment (compare with fig. 15).

Fig. 33 Model of an implanted girdle from individual Tr. d/2 4 which developed from the transplantation, along with the dorsal portion of the limb mesoderm, of the dorsal zone of the girdle rudiment (compare with fig. 16).



INHERITANCE OF COAT-COLOR IN CATS

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TWO PLATES

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I. INTRODUCTION: THE COLOR FACTORS OF DOMESTIC CATS

In a series of experiments begun at the University of Pennsylvania in the autumn of 1914 and extending up to the present time, the inheritance of coat-color in cats has been investigated. Although the number of litters obtained has not been large, it has been found possible to determine several points in regard to the mechanism of heredity by means of critical crosses. This has been largely due to the fact that the characters studied segregate for the most part cleanly from each other so that it has been easy to classify the animals.

My thanks are due to Dr. McC'lung and to Dr. Colton for the kindly interest which they have taken in the work and to the

University of Pennsylvania for the expense of the experiments. I also wish to thank the Zoological Society of Philadelphia for the opportunity of crossing my cats with the Caffre cat.

Before presenting the data and discussing the inheritance of the various characters in detail it may be well to name and to define briefly the factorial differences involved.

The ticking or agouti series contains at least two and probably more different factors. Black, non-agouti, or uniformity, *a*, is a recessive allelomorph to agouti, *A*. An extreme amount of ticking is frequently observed, and this represents, in all probability, a third allelomorph in the series. Results are not conclusive, but are consistent with such a hypothesis. This extreme ticking may tentatively be called *A'*.

The banding or tabby series contains at least two and probably three different allelomorphs. They may be called lined, *B'*, striped, *B*, and blotched, *b*. The allelomorphism of these is again merely tentatively assumed, but I believe there is good reason to consider them as a triple allelomorphic series. The agouti and the banding factors will be discussed fully later in this paper. It may merely be stated here that all cats are either lined, striped, or blotched and that there are no intergrades. The character of the banding is, however, easily recognized only in the presence of ticking. In the black or non-agouti series banding exists as 'ghost patterns' recognizable usually but not always in the fur of kittens, and only occasionally distinguishable in full-grown cats.

Intense pigmentation, *M*, is a simple dominant over dilute or maltese, *m*.

White, *W*, is a simple dominant over color, *w*.

Yellow, *Y*, is allelomorphic with black, *y*. This locus is sex-linked and shows the male to be digametic. The heterozygous female is *usually* yellow-spotted or tortoiseshell, but may range in color to solid black or solid yellow.

White-spotting is very irregular and probably depends upon many factors. It will not be considered in detail in the present paper. It appears to segregate independently of the factors above mentioned.

Albinism, as we have it in the rodents, is apparently un' nown in cats. It is possible that Siamese dilution represents an approach toward this condition.

Variations in the tone of coloration are extensive, but apparently not clearly segregating. Silvers represent a reduction of yellow pigment and also of black. Smokes are very dark silvers. The lighter bands of tabbies are straw- or cream-colored, varying to white in silver tabbies and brown in brown tabbies. Occasionally the brown varies to a rusty red. Silver creams are yellow cats in which the yellow pigment is reduced to a minimum so that the hair sometimes appears almost white. According to fanciers, silver tabbies bred together occasionally throw brown tabbies.

II. PRESENTATION AND DISCUSSION OF DATA

A. Maltese dilution

Maltese dilution appears to be a simple Mendelian recessive. It apparently exists in combination with all other factorial differences, but I have not as yet seen its representative in the lined or narrow type of banding. It is always sharply distinguishable from black, but shows considerable variation in its own intensity. It is to be compared to slaty-blue in the mouse, the rabbit, and the dog. No corresponding color is known in the rat or in the guinea-pig. Cream or dull yellow is its corresponding color in the yellow series; blue and cream, in the tortoiseshell.

Five litters from dilute by dilute gave sixteen dilutes—eight males and eight females.

Six litters from intense by intense gave twenty-four intense—sixteen males and eight females.

Five litters from intense males by dilute females gave five intense males, nine intense females, and one dilute male.

Eight litters from dilute males by intense females gave nine intense males, three intense females, nine dilute males, and eleven dilute females.

These data show merely that maltese dilution is not sex-linked.

B. White and white-spotting

White-spotting in cats is exceedingly irregular in amount and distribution, but tends to appear more commonly on the under parts. There appears to be no regularity in dominance and probably many factors are involved. The degree of white-spotting in the parents tends to appear again in the offspring, although wide segregation occurs. I have usually selected cats with relatively small amount of white and have obtained kittens of the same general character.

Solid white appears to be a complete dominant over color whether the color is self or spotted with white. It is possible that it may be allelomorphic with one or more of the white-spotting factors, but my data are not conclusive on this point. Castle ('16) regards it as possibly an extreme form of white-spotting.

A cream male (8)¹ with slight amount of white crossed to a blue-eyed deaf white female (7) sired (6) three pure white kittens; one male and two females. The kittens had normal hearing and developed yellow eyes. The male had a slight smuttiness of the hairs on top of the head which appeared when he was two weeks old and then shortly disappeared. The females never developed any pigment in the hair. The same pair of cats mated again and produced two solid white kittens which were not reared.

The cream male (8) was later mated (32) to a yellow-eyed white with normal hearing (22). Two whites, a male and a female, were produced, as well as two females that were entirely self color, a blue and cream and a cream. I was unable to detect any white hairs on these two pigmented kittens. It would appear, then, that the white mother carried factors which dominated the slight white marking of the father, and thus produced totally self-colored kittens.

¹ Individual numbers have been inserted after mention of any animal that is referred to more than once in this paper. Matings have been numbered similarly. It will thus be possible for the reader to check up the genetic composition of any animal by its progenies from various matings.

Dr. Little reports a mating (43) of a yellow-eyed white male by a cream and white female. A single yellow-eyed white female was produced.

A mating (35) of a blue-eyed white Angora male (16) by a yellow-eyed short-haired white female produced three solid whites and a black that was self-colored except for very small white spots between the legs. A mating (36) of the same male by a maltese female produced a solid white and a near self black.

Besides these I have three records of blue-eyed white females which produced both colored and white offspring. The male parents were unknown. One produced a solid white yellow-eyed female and a solid black male. One produced in two litters six solid white and three almost entirely black. The third produced a solid white and an intense striped tabby with belly, nose, breast, and feet white.

Davenport ('05) reports a mating of a black Manx male by a blue-eyed, deaf, white female, which produced two whites, one black, one tiger, and one maltese.

The interesting correlation of blue eyes and deafness with white coat is not yet satisfactorily explained. Dominance of eye color seems very irregular. I am informed by breeders of white cats that yellow-eyed by yellow-eyed may produce blue-eyed, and also that blue by blue may produce yellow. Odd-eyed cats also frequently appear in these crosses.

Przibram ('08) reports experiments with odd-eyed white Angora cats. Results were very irregular. The cats bred true to whiteness. As regards correlation of deafness and blue eyes, he says,

Von Interesse ist es, dass, soweit eine Prüfung des Gehörs unternommen werden konnte, die blau-blauen erwachsenen Katzen alle völlig taub waren, was ja mit früheren Beobachtungen von Darwin und Rawitz übereinstimmt; die asymmetrischen Augenfarben scheinen dem ganz entsprechend mit einer halbseitigen, die Seite des blauen Auges betreffenden Taubheit betroffen zu sein. Die Correlation zwischen blauen Augen und Taubheit bleibt also auch bei der asymmetrischen Vererbung bestehen. Dabei bleibt die Correlation zwischen 'blauen Augen' der Katzen und 'Taubheit' auch für die Körperhälfte bestehen.

Dr. C. C. Little showed me a black and white cat with odd eyes. The hair surrounding the blue eye was white, while that about the yellow eye was black. Blue eyes in pigmented cats are rare, except of course in the case of the Siamese.

I would suggest, therefore, as a working hypothesis that the incidence of white-spotting in connection with the dominant white factor produces the blue eye, or in other words a 'white spot' about the eye of a white cat makes the eye blue, while a 'pigmented spot' about the eye of a solid white cat makes the eye yellow. It may be also that a 'white spot' in the ear of a white cat makes it deaf. This would explain why it is so difficult to get blue-eyed white cats with normal hearing as it would be difficult to localize the 'white spot' upon the eye and to keep it away from the ear. This may also explain why odd-eyed cats are frequently defective in hearing only on the side having the blue eye, as noted by Przibram. It would not be a difficult matter to test this hypothesis.

C. Solid yellow and yellow-spotting

The tortoiseshell cat has been the subject of much interest and discussion in genetic literature dealing with sex-correlated phenomena.

Doncaster ('05) considered the problem and tried to explain the peculiarities of inheritance by variations in dominance. Little ('12) suggested the hypothesis of a single sex-linked pair of allelomorphs with the male digametic. He used the term 'sex-limited character,' which has since been restricted to simple Mendelian heredity in which sex reverses the dominance of the allelomorphs. Doncaster ('12) accepted Little's suggestion as in general satisfactory, but pointed out that occasionally black females are produced from matings of black female by yellow male. According to Little's hypothesis, the females should always be tortoiseshell from the reciprocals of black by yellow and the males should be like the mother, disregarding of course dilution, tabby, etc. Doncaster suggests an occasional break in sex-linkage to explain these anomalous blacks, as also the

occurrence of the rare tortoiseshell male. In 1913 he gave a further discussion of the subject and an excellent summary of data collected from fancy breeders. In 1914 he suggested non-disjunction of the sex-chromosomes in oögenesis to explain the matroclinous black females. These explanations are all more or less unsatisfactory for one reason or another, as admitted by Doncaster and by Little.

I have pointed out (1915) that the hypothesis of simple sex-linkage first suggested by Little may be sufficient to account for the conditions if it be considered that the heterozygotes, which must be females, vary from black through various degrees of yellow-spotting to solid yellow. In the male, presumably, conditions are much more stable, as it is impossible to have a heterozygote. Thus I have suggested that a gametically yellow male ($YX -$) may become tortoiseshell by extreme selection of black extension factors, while a gametically black male ($yX -$) may become tortoiseshell by an extreme selection of yellow extension factors. The possibility is of course not excluded that there may be a single factor or particular combination of factors that produces yellow-spotting in the male.

Ibsen ('16) has suggested close coupling of two pairs of sex-linked allelomorphs, and attempts to explain anomalies by crossing-over. This does not account for all the results, however, as he himself points out.

The data concerning the tortoiseshell problem which I have gained from my experiments are as follows:

A long-haired cream male (8) ($a.b.m.Y$)² was crossed (12) with an intense striped tabby (25) ($A'B.M.y$). There resulted one maltese ($a.B.m.y$) and two intense striped tabby ($A'B.M.y$) males and one blue and cream ($a.B.m.Yy$) female.

The same male was crossed twice (9 and 30) to a black (20) ($a.B.M.y$). There resulted three black ($a.B.M.y$) and two maltese ($a.B.m.y$) males and two orange and black ($a.B.M.Yy$) and three blue and cream ($a.B.m.Yy$) females.

² The genetic formula includes only factors known to be present. The duplex condition is not expressed except in the case of the sex-linked pair $Y-y$ in which dominance is variable.

The same male was crossed (33) to an intense striped tabby (3) (A.B.M.y). There resulted one intense striped tabby (A.B.M.y) male and one blue and cream (a.b.m.Yy) female.

The same male was crossed (13) to a maltese (17) (a.b.m.y). There resulted one maltese (a.b.m.y) male and two blue and cream (a.b.m.Yy) females. These blue and cream females are of interest as they are almost anomalous blacks. They were mentioned by me in a previous paper (1915, p. 519). They are almost entirely dilute blacks except for a very restricted amount of white on the under parts. One has a very small amount of cream bordering the white at a few points, and a small cream spot on the back. The other is entirely without cream except for a few cream hairs on one leg and at the tip of the tail. These kittens then represent a very close approximation to the anomalous black.

A short-haired cream male (24) (A'.b.m.Y) was crossed twice (34 and 49) with an intense striped tabby (3) (A.B.M.y). There resulted five males—three maltese (2 a.B.m.y and 1 a.b.m.y), one black (a.B.M.y), and one intense striped tabby (A'.B.M.y); and four females—two blue and cream (a.b.m.Yy), one orange and blotched (A.b.M.Yy), and one blue and cream striped (A'.B.m.Yy).

Summarizing matings of 'yellow' male (YX -) by 'black' female (yX yX), we have seven matings giving fifteen 'black' males (yX -) and thirteen 'tortoiseshell' females (YX yX).

Crosses of yellow male by tortoiseshell female are as follows:

A cross (37) of an orange striped male (B.M.Y) by a blue and cream female (26) (a.b.m.Yy) produced two orange-striped females (B.M.Y).

A short-haired cream male (24) (A'.b.m.Y) was crossed (44) with an orange and black female (28) (a.M.Yy). There resulted one cream (m.Y) male and three females—two cream (m.YY) and one blue and cream (a.m.Yy).

The same male was crossed (45) with another orange and black female (30) (B.M.Yy). There resulted three males—one black (a.M.y), one blotched maltese (A'.b.m.y), and one cream (B.m.Y).

The same male was crossed (47) with a blotched blue and cream (13) (A.b.m.Yy). There resulted two males—one maltese (a.b.m.y) and one cream (b.m.Y); and three blotched blue and cream (A.b.m.Yy) females.

Summarizing crosses of 'yellow' males (YX -) by 'tortoiseshell' females (YX yX), we have four matings giving three 'black' males (yX -), three 'yellow' males (YX -), four 'yellow' females (YX YX), and four 'tortoiseshell' females (YX yX).

The long-haired cream male (8) (a.b.m.Y) mentioned above was crossed (32) to a yellow-eyed white cat (22). There were produced one white male, one white female, one cream female (B.m.YY), and one blue and cream female (a.B.m.Yy). Since a cream kitten as well as a blue and cream was produced, it is probable that the yellow-eyed white was gametically a tortoiseshell.

Dr. C. C. Little has very kindly supplied me with data in regard to an anomalous cream female (23) which breeds like a tortoiseshell. This female he has given to me along with three of its offspring. She has, while in my possession, produced four kittens by her cream son. They are two cream females, one cream male, and one maltese male. The maltese male would of course not be expected from a mating of two yellows. The under parts of the anomalous female are white. The upper parts are entirely cream and show the blotched pattern very plainly. No trace of black pigment can be found, although I have examined samples of the hair from various parts of the body under the microscope.

The following gives in detail the offspring from this anomalous cream female. The unexpected progeny are recorded in italics.

When crossed with (42) a dilute blotched male (A.b.m.y), she produced two males—one *blotched maltese* (A.b.m.y) and one *maltese* (a.m.y); and one blotched blue and cream female (A.b.m.Yy).

When crossed with (39 and 40) an intense striped male (A.B.M.y), she produced in two litters two males—one orange

(M.Y) and one cream (24) (A'.b.m.Y); and two females—one *black* (a.M.yy) and one orange and black (a.M.Yy).

When crossed with (41, 46, and 50) her cream son (24) (A'.b.m.Y) she produced in three litters three males—one *blotched maltese* (A.b.m.y), one *maltese* (a.b.m.y), and one cream (m.Y); and two cream females (m.YY).

When crossed with (38) an orange-striped male (B.M.Y), she produced one *black male* (a.M.y) and three females—two striped orange (B.M.YY) and one *striped orange and black* (a.B.M.Yy).

Summarizing the matings of this cream and white female, we find that when bred to 'black' males (yX -) she produced two 'black' males (yX -), two 'yellow' males (YX -), two tortoiseshell females (YX yX), and one *black female* (yX yX).

When bred to 'yellow' males (YX -), she produced three 'black' males (yX -), one 'yellow' male (YX -), four 'yellow' females (YX YX), and one *tortoiseshell female* (YX yX).

Of these matings Dr. Little says: "The dilute yellow and white female is interesting because she forms gametes carrying black and breeds exactly like a dilute *tortoiseshell* and *white* animal, although there is no trace of black pigment anywhere on her." She is then an anomalous yellow. Dr. Little further states: "Dilute yellow, like the same color in mice, does not depend upon the depth of color, but is essentially a *dull* yellow ranging anywhere from intense pigmentation to dilute cream color." It is of course relatively not as intense as the orange. It is possible that this variation in cream color is due to the same factors which produce the variations towards silver in tabbies and others.

Yellow-spotting in cats may be compared essentially to the same condition in guinea-pigs. In the latter there is great range of variability as in the former. In cats, however, one of the allelomorphic pairs determining black or yellow extension is much more potent than the others and is sex-linked. The heterozygous female (YX yX) represents a much more unstable condition as regards spotting than either of the homozygous females or than either of the haploid males, for in the heterozygote the factors yellow, Y, and black, y, are balanced against each other.

The sterility of the tortoiseshell tom has frequently been remarked upon. Cutler and Doncaster ('15) discuss this question and show drawings of sections of the testis of a sterile cat of this sort. Normal reproductive cells are altogether lacking. In summarizing the data on sterility of male tortoiseshells, they find that one was certainly fertile, two completely sterile, one almost if not quite sterile, and two doubtful. It appears, then, that sterility may be highly correlated with yellow-spotting in the male.

The black-yellow allelomorphic pair in cats is of particular interest, as it is the only case of sex-linkage known in mammals, other than the sex-linked defects of man.

D. Siamese dilution

Bateson ('13) says of the Siamese cats: "These animals, which breed perfectly true, were introduced from Siam, where they have been kept for an indefinite period as pets of the royal household. Like the Himalayan rabbit, Siamese cats are born almost white, but the fur becomes a curious fawn with darker chocolate points on the ears and extremities." Crosses of Siamese by other cats are cited by Weir ('89). Quoting from a Mr. Young, he says (p. 76), "They invariably showed the Siamese cross in the ground color." But Lady Dorothy Nevill says, "None showed any trace of the Siamese, being all tabby."

Two pregnant females of common cats brought into the laboratory produced kittens of a peculiar ashy color with darker extremities. The kittens resembled very closely adult Siamese cats.

One of the pregnant females, a maltese (5) (a.m.) produced (15) two females which were ashy, with nose, ears, feet, and tail slightly darker, and two females and two males which were ashy with black extremities. A record taken fifty days after birth showed that the lighter kittens had become maltese, while the kittens with black extremities had become steel colored or almost black. They later became completely black. Ghost patterns were seen on four of the kittens, but unfortunately a critical

examination was not made of the other two. Ghost blotched was very much accentuated by the ashiness, but ghost striped did not appear especially so. It is probable for this reason that the two doubtful ones were ghost striped. Of the other four the maltese was ghost striped, and the three steels were ghost blotched.

The other pregnant female (2) was a black and white (a.M). She produced (4) four kittens—two black females upon which no record of ghost pattern was made, an ashy female with black extremities, and an ashy male with dark but not black extremities. Sixty-six days after birth the ashy female had developed into a steel black which clearly showed ghost-blotched pattern, and eighty days after birth the ashy male was maltese with ghost-blotched pattern very evident.

Unfortunately, the inheritance of this peculiar ashy color could not be followed out at the time the kittens were on hand. I am, therefore, unable to say whether it represents the heterozygote for Siamese dilution.

E. Banding and ticking

a. Statement of factorial differences and description of characters. Ticking or agouti in cats, as in rodents, is characterized by yellow bands on the hairs. It increases with age so that kittens are relatively less ticked than cats. I have tentatively considered the agouti factors as a series of triple allelomorphs— A' , much ticked, A , little ticked, and a , non-ticked, with dominance of A' over A and a , and of A over a .

The banding factors, I have also represented as triple allelomorphs— B' , lined, B , striped, and b , blotched. These factors affect the formation of yellow pigment, in a yellow cat (A or a.B.M.Y) forming bands of straw color alternating with orange. In a tortoiseshell tabby cat (A .B.M.Yy) the orange bands in the 'yellow spots' are continuous with the black bands in the 'black spots,' while the straw-colored bands are continuous through both regions. In a tortoiseshell (a.B.M.Yy) alternate banding of straw and orange shows clearly in the 'yellow spots,' while the

'black spots' are uniform black. The same condition obtains in the case of maltese dilution, but the contrast in the bands is not as obvious and there is general reduction in the amount of yellow pigment.

Uniformity or lack of banding in yellow cats is apparently due, as has been pointed out to me by Dr. Sewall Wright, to some other condition than the lack of the agouti factor. As regards the existence of such cats, Mrs. Leslie Williams ('08) writes: "The self-orange Persian is more of an ideal than a reality, for it is actually a red tabby without the tabby markings, and at present it is a case of 'more or less,' the upshot being that the least marked cat in the class takes the prize."

Silvering is a general reduction in the amount of yellow pigment. The straw bands of tabbies then become white. Figure A shows a silver-striped tabby skin. Black stripes alternate with white. In the skin shown in figure B, on the other hand, there is a considerable amount of yellow pigment. A striped tabby (9) brought into the laboratory pregnant had lighter bands of a decidedly reddish color. This apparently represents the opposite extreme of variation from silvering. Intense black stripes alternated with rusty red. She gave birth (22) to three male kittens—one striped with black and red; one blotched with black and red, and one striped with black and straw color. Here, then, is an indication that the extreme reddish tone is hereditary.

For an understanding of banding we may first consider figure B. The skin shown here is from a striped tabby male (7.2) forty-two days old. It may be seen that the bands run longitudinally along the back and are most easily seen near the mid-dorsal line posteriorly. On the sides the bands are transverse and tend to be broken into spots. We may think of this condition as having been produced by longitudinal and transverse waves of pigment-forming metabolic activity. The longitudinal waves form transverse bands. The areas of greatest activity form orange bands in yellow cats, while in tabbies these bands are black. The areas of less activity form, of course, the lighter bands. The transverse waves appear to originate at the mid-dorsal line. They form longitudinal bands on the back. As

they pass outward and down the sides, the areas of greater activity tend to thicken the transverse bands. In the areas of less activity the transverse bands are often evanescent. It thus appears that black or orange spots, in tabbies or yellows, respectively, are produced in the regions of greatest metabolic activity.

The ticking and the banding factors *appear* to act in the same regions, and thus the ticking reveals the straw-colored rather than the orange bands. Agouti is, however, in all probability uniform over the body surface in cats as in rodents. This matter will be discussed in detail after the presentation of data.

The skin shown in figure B had a high degree of ticking, and thus shows the longitudinal bands clearly. The cat shown in figure A is less ticked and the increased amount of black pigment on the back obscures the longitudinal bands. Figure D shows a very dark-striped tabby. While the bands on the sides are clearly seen, the longitudinal bands are obliterated by the black pigment. The cats shown in figures B and D are from the same litter and represent extreme segregation of ticking.

As has been said, ticking increases in cats as in rodents with maturity. The same kitten may, therefore, show different degrees of it at different ages. It is thus necessary to consider age in making comparisons with respect to this character. Ticking always segregates sharply from black. Various degrees of ticking ranging from that shown in figure B to that shown in figure D, however, occur. I have classified the extremes tentatively as A' and A, but their allelomorphism with a is uncertain. There may be intermediate allelomorphs or the variations may be due to modifiers.

The blotched pattern is shown in figures F and H. Figure F is from a kitten extremely ticked at birth. Such a kitten develops into a cat that has yellow in all of its hairs. The black bands of the kitten become ticked in the adult. The lighter bands become entirely straw-colored. We have in this extreme ticking an approach toward the sooty yellow, as in the mouse.

The skin shown in figure H is from a kitten one week old. Nevertheless, it is much darker than that shown in figure F. Such a kitten develops into a dark-blotched adult. The ticking

increases with age until the cat appears much like the kitten shown in figure F.

For a discussion of the blotched pattern in comparison with the striped, the degree of ticking shown in figure F is most favorable. The bands shown here are broad and consequently not as numerous as in the striped. A median dark longitudinal band down the back is cut just behind the shoulders by dark and light transverse bands. The alternation of dark and light bands is not as obvious in the blotched pattern as in the striped, since the bands are relatively wide and the longitudinal and transverse bands interfere with each other. The tendency of the bands to become broken into spots or blotches may be explained in the blotched, as in the striped, by a conflict of longitudinal and transverse waves.

The lined or narrow-banded pattern is rarely seen in cats in this country. The bands are extremely narrow and frequent and are best seen when the hair is very short and the ticking is of just the proper degree. Figure C shows a rather dark-lined cat (28.3) forty-five days old. The narrow banding shows clearly about the edge of the skin and to some extent on the sides. Such a cat becomes somewhat lighter when it grows older. It is very dark when young and appears black and tan like figure G, which is from a lined kitten (19.3) one week old. Narrow bands are seen in the tan areas of the latter and the back and sides show narrow bands when the skin is turned in certain relations to the line of vision. These narrow bands are really a 'ghost pattern' comparable to the 'ghost patterns' of striped and blotched seen in fully black cats. They may be seen in the fur running transversely down the sides. On the skin they may be seen running in the same way and also longitudinally down the back. They are much narrower and more numerous than the bands of striped cats. Lined cats occur in Africa and to some extent in Europe. They are known as African, Caffre, or Abyssinian cats.

In black and maltese kittens 'ghost patterns' are seen clearly in the skin and are not difficult to recognize in the fur. As the kittens become older the ghost patterns sometimes show more clearly in the fur for a time, although they disappear from the

skin. In adult cats ghost patterns are occasionally seen. I have been able to classify all black or maltese kittens as either striped or blotched. A lined cat lacking agouti has not yet been obtained, but this I am hoping to do in time by the proper crosses.

Figure E is from the skin of a lined kitten at birth. It is an extremely ticked example and would probably have grown to a sooty yellow adult. The back is black, but well scattered with ticked hairs, thus differing from the skin shown in figure G. The transverse bands are shown about the edge of the skin at the sides and about the tail. The longitudinal bands are suggested by two ticked spots at the back of the neck. Just posterior to these spots are two parallel ticked lines. On the body near the tail may also be seen longitudinal bands.

Fundamentally, then, the lined, the striped, and the blotched patterns are comparable, differing only in the width of the bands.

A pair of lined cats is owned by the Zoological Society of Philadelphia. The male is dark while the female is much lighter. A comparison of the degree of ticking of the two may be of interest. The back of the male is black, the sides very dark showing narrow ticked bands. The back of the female is dark but ticked and grades into sooty yellow on the sides, showing no dark banding. The banding on the head and breast of the male is for the most part black, while in the female it is brown shading to sooty. In the male the back and end of the tail are black, while ticked rings are seen only toward the base. In the female the entire tail is ringed with sooty yellow. In both animals the feet are sooty yellow, the soles black. In the male the black bands of the sides extend down the legs to the feet, while in the female the sooty yellow of the feet extends well up on the legs.

b. Experimental data. When bred together (19) the lined cats produced four kittens—a dark-lined male, the skin of which is shown in figure G; a dark-blotched male, the skin of which is shown in figure H; a dark-blotched female, and a light-lined male (21). The last-mentioned animal has been raised and is now in the possession of Dr. Charles Penrose, of Philadelphia. It has been examined by the writer, who finds that at the age

of two years and three months it is in color almost the exact counterpart of its mother. It has, therefore, received from its mother the factor A' while its three sibs have received the factor A for which the mother is presumably heterozygous, unless indeed she is carrying non-agouti, a . The cross may be expressed: $AAB'b \times A'AB'b = 1 AAB'? + 1 A'AB'? + 2 AAbb$. Whether or not the two lined offspring are homozygous for B' or carry b is unknown.

The dark-ticked lined male was bred to a very much ticked blotched female (14). This female had been brought in pregnant and had produced (11) two blacks showing ghost striped, one blotched, and two striped. One of the striped had a *very high* degree of ticking. The skins of the others were not saved and no determination of the exact amount of ticking was made at the time. When bred to the dark-ticked lined male this female produced four kittens—one lined and three blotched. The lined is shown in figure E and one of the blotched in figure F. The other two blotched were similar in amount of ticking to that shown in figure F. This female may then be considered as of formula $A'abb$. The cross to the lined male, $AAB'b$, gave 1 $A'AB'b$ and 3 $A'Abb$. Larger numbers would probably have shown some dark-ticked kittens.

The same male was bred to the black and white female (2) mentioned under the discussion of Siamese dilution. She had produced (4) by an unknown male two ghost blotched kittens—a black and a maltese—and two blacks in regard to which no ghost pattern was recorded. When bred to the lined male she produced (29) four kittens—three lined and one blotched. All the skins were kept and all were very dark. The mating may therefore be represented: $\sigma AAB'b \times \varphi aabb = 3 AaB'b + 1 Aabb$.

The lined male was also bred to a black female (10) which showed indistinct stripes in her fur. She had produced (23) from an unknown male four black males, all showing stripes as ghost pattern. When bred to the lined male she produced (28) one lined and two striped kittens. The lined is shown in figure C and one of the striped in figure A. The other striped was

about the grade of ticking as that shown in figure A. If my hypothesis of the allelomorphism of the agouti factors is correct, these striped kittens must represent a light variation in the dark agouti. In the case of these kittens the light color may be due to silvering, as there is practically no yellow in the fur of either of the striped, although the lined shows a fair amount. The cross may be represented: σ^7 AAB'b \times φ aaBB = 1 AaB'B + 2 AaBb.

A highly ticked blotched male (11) was crossed (14) with a black female (15). Four highly ticked blotched kittens similar to figure F resulted. The cross may be represented: σ^7 A'A'bb \times φ aabb = 4 A'abb. The same male was later crossed (25) with a maltese striped female (4). Three intense striped and two intense blotched resulted. Unfortunately, the grade of the ticking was not determined.

A very dark striped female (3) was crossed (16) to a very dark blotched male (6). Six kittens were produced—two blotched, three striped, and one black showing stripes as ghost pattern. The blotched and two of the striped were dark. The other striped was slightly lighter. It was not, however, as light as those that are grouped as much-ticked. It shows clearly, nevertheless, that ticked cats may produce offspring apparently more ticked than themselves. The cross may be represented: σ^7 Aabb \times φ AaBb = 3 AA or AaBb + 2 AA or Aabb + 1 aaBb.

The same dark-striped female (3) was crossed (33) with a long-haired cream male (8) which appeared from other crosses to be homozygous for blotched and for non-agouti. The two kittens were a blue and cream female showing blotched as ghost pattern and a dark-striped male. The latter has grown up and shows exactly the same dark coat-color as his mother. The cross may therefore be represented: σ^7 (aa.bb.mm.Y-) \times φ (Aa.Bb.-Mm.yy) = 1 σ^7 (Aa.Bb.Mm.y-) + 1 φ (aa.bb.mm.Yy).

The dark-striped female (3) was mated twice (34 and 49) with a short-haired cream blotched male (24) and produced nine kittens as follows: Males, 1 a.B.M.y., 2 a.B.m.y., 1 A'.B.M.y., 1 a.b.m.y. Females, 1 A'.B.m.Yy., 2 a.b.m.Yy., 1 A.b.M.Yy.

The light-ticked kittens probably inherited the factor A' from their yellow father. That the male carries the factor A' is shown by the fact that when crossed (45) with a black and orange (30) there were produced three males—a black and white in which there was so much white that it was impossible to determine the ghost pattern, a cream, showing stripes, and a blotched maltese in which the ticking stood out extremely clearly. The latter is being raised and differs decidedly in amount of ticking from many other blotched maltese cats in my possession.

The long-haired cream male (8) was crossed twice (9 and 30) with a black female (20). Ten kittens were produced, all of them showing stripes as ghost pattern. The female is therefore $aaBB$. The cross may be expressed: $\sigma aabb \times \varphi aaBB = 10 aaBb$.

The same male (8) was crossed (12) to a much ticked striped female (25). Of the four kittens produced two were non-ticked and showed stripes as ghost pattern and two were much ticked and striped. The cross may therefore be expressed: $\sigma aabb \times \varphi A'aBB = 2 aaBb + 2 A'aBb$.

The same male was crossed (13) to a maltese female (17). Three dilute kittens resulted, all with blotched ghost pattern. $\sigma aabb \times \varphi aabb = 3 aabb$.

It is clear, then, that the cream male (8) does not carry the ticking factor, for when crossed with two non-ticked females it has sired thirteen non-ticked kittens. It also probably is homozygous for blotched, although it does not show the pattern, for when crossed to a maltese female it sired three blotched kittens.

It thus appears that the long-haired cream (8) does not carry agouti and does not show the blotched pattern, while the short-haired cream (24) carries strong agouti, A' , and shows the pattern. Whether the agouti factor tends to bring out the pattern more strongly in a cream may yet be an open question. I am inclined to think that the pattern is obscured to some extent in long-haired cats by the length of hair.

An orange-striped male was crossed (37) to a blue and cream female (26) that showed ghost blotched pattern. Two orange-striped females resulted. The cross may be expressed: $\sigma (BB.Y-) \times \varphi (bb.Yy) = 2 (Bb.YY)$.

c. The number of loci involved. The facts thus far collected, then, are consistent with the assumption of two loci, one for the banding factors and one for the ticking factors. Since, however, all variations from very light to very dark ticking occur and since dark-ticked cats heterozygous for black may produce kittens of somewhat lighter grade than themselves, it is probable that factors at other loci recombine to modify the ticking. Tests are now being made which, it is believed, will determine definitely whether light ticking and dark ticking are both allelomorphic with the same factor for black; that is, whether they form a triple allelomorphic series.

The three types of banding, lined, striped, and blotched, are each entirely distinct. No intergrades have been observed. The natural assumption is to suppose that they form a triple allelomorphic series, B' , B , and b , as I have tentatively assumed. But if two loci are involved the conditions might be expressed as follows: A lined cat might be $LLBB$, $LLBb$, $LLbb$, $LlBB$, $LlBb$, or $Llbb$. A striped cat might be $llBB$ or $llBb$. A blotched cat would then be the double recessive, $llbb$. This scheme apparently fits the genetic data thus far collected. Striped and blotched would act as a pair of simple allelomorphs, B and b . The crosses involving the lined cats would be expressed by supposing that they are both of formula $Llbb$. Bred together they produced lined, $LLbb$ or $Llbb$, and blotched, $llbb$. Crossed with blotched, either black or ticked, $llbb$, they give lined, $Llbb$, and blotched, $llbb$. Crossed with homoyzous striped, $llBB$, they give lined, $LlBb$, and striped $llBb$.

The same crosses for testing the agouti factors will also test the allelomorphism of the banding factors. Other combinations that are being made with lined should give yellow and maltese lined.

d. Physiology of color-production. Reference should now be made to Wright's ('17) papers on color inheritance in mammals. Wright classifies color factors according to their effects on either one of two enzymes. Enzyme 1 is the basic enzyme for color production which, acting alone on chromogen, produces yellow. Enzyme 2 is supplementary to enzyme 1. It has no effect alone

either on chromogen or on yellow pigment. Combined with enzyme 1 it oxidizes chromogen to sepia.

The agouti factors are considered as determining an inhibitor of enzyme 2. "Factor A determines the production of an inhibitor with the same subtraction effect on enzyme 2 everywhere." This inhibitor acts in waves along the individual hairs. The regions of greatest concentration determine the yellow bands, while those of less concentration are black.

In yellow cats it is seen that banding occurs over the surface of the body, straw-color alternating with orange. Banding, therefore, affects enzyme 1. In black cats the bands are almost indiscernible. There is, then, enough of enzymes 1 and 2 generally distributed to produce a uniform black. In the presence of the agouti factor, however, yellow bands appear in the individual hairs. These bands are much wider in the areas corresponding to the straw-colored bands of yellow cats. In fact, the black may be here entirely obliterated. The hairs in the areas corresponding to the orange bands of yellow cats are much darker and may be without apparent ticking. It therefore appears that the banding factors affect enzyme 2, for if enzyme 2 were uniformly distributed as in rodents, the agouti factor should cause a uniform ticking over the body surface, not an alternation of dark and light bands.

The banding factors may be thought of, then, as determining waves of general metabolic activity affecting both enzyme 1 and enzyme 2. In the black cat the regions corresponding to the orange bands in the yellow cat would be a dense black, a sort of black dominant to agouti, comparable to Punnett's ('12 and '15) dominant black in rabbits, while the regions corresponding to the straw-colored bands would be comparable to ordinary black in being recessive to agouti.

For helpful criticism and discussion of these matters I am much indebted to Dr. Wright, whose papers I have already mentioned.

III. THE ORIGIN OF COLOR VARIETIES OF THE CAT

It is generally assumed that the domestic cat is polyphyletic in origin. Darwin considered this to be the case. Keller ('02) discusses the matter and agrees with Darwin on this point. Elliot ('83) believes that the cat is descended from a number of wild species and supposes that it has crossed at various times with small wild cats in different countries. He attempts to trace the well-known color variations as well as variations in form to such hybridizing.

Rope ('81) and Pocock ('07) both recognize the characters blotched and striped and believe that all cats, whatever their color, fall in one or the other of these two classes. Pocock states:

It is needless to say more in support of the contention that if a decided difference in the 'pattern' of Domestic Cats exists, it must be regarded as furnishing a surer basis for their classification than the length of hair, the tint of the coat, or the stunting of the tail. It may also be claimed with assurance that the pattern supplies a more important clue to the ancestry of the Domestic Cat than the features just mentioned. . . . Frequently at all events the so-called 'blotched' pattern can be detected in certain lights even in 'Whites' and 'Blacks.'

Pocock also recognized the lined variation, called by him Abyssinian.

Cats of the so-called 'Abyssinian' breed may be descended, for anything I know to the contrary, from specimens of *F. ocreata* directly exported from Abyssinia. They are certainly not unlike some self-coloured examples of that species. On the other hand, it would, I imagine, be difficult to separate them from fulvescent 'Ticked' Cats, which appear to me to be nothing but examples of the torquata-type in which the pattern is broken up and evanescent.

The torquata type is what I have called striped. Pocock discusses the synonymy of wildcats and of the domestic cat. The whole matter appears to be much confused.³

³ The wildcat of Europe is usually called *Felis catus* L., but inasmuch as Linnaeus' description agrees with the blotched pattern while the European wildcat is striped, it is considered by Pocock and others that Linnaeus was referring to the domestic cat. *Felis sylvestris* Schreber is therefore chosen as the name for the European wildcat. Of the striped form Pocock says: "To feral or do-

Dr. A. Nehring ('87) believed that the cat has a dual origin, being descended from a domestic Chinese cat and from the Egyptian cat, *Felis maniculata*. The origin of the striped pattern is easily traced to the European wildcat or to the African wildcat. Of the blotched type Richard Lydekker (*Encyclopaedia Britannica*) says:

It may be suggested that the blotched tabby type represents Dr. Nehring's presumed Chinese element in the cat's parentage, and that the missing wild stock may be one of the numerous phases of the leopard-cat (*F. bengalensis*), in some of which an incipient spiral arrangement of the markings may be noticed on the shoulder.

The attempt is made by many authors to trace all variations in morphology and color to some wild ancestor. To do so appears to me unnecessary, as all such variations might occur under domestication. The strictly domestic color variations in the cat may be considered maltese, white, white-spotting, yellow, and Siamese dilution. Such variations, if they occur in nature, appear to be blotted out, as they are certainly not characteristic of any wild species. They occur in numerous domestic animals and may be said to be variations by which domestic species 'mimic' each other.⁴

mesticated examples of this cat have been given many names, of which *torquata* is the best known and *angorensis* or *striata* possibly the oldest. . . . It closely resembles in pattern two existing species, namely, the so-called Egyptian cat (*F. ocreata*) and the European wildcat (*F. sylvestris*)."

Pocock thinks that the blotched or *catus* type is derived from some extinct, probably Pleistocene cat of western Europe.

Pocock uses the term *torquata* for striped and *catus* for blotched, which is just the reverse of many other authors.

There is, further, a so-called *Felis torquata* of India that is considered by some to be related to the spotted desert cat (*F. ornata*).

Inasmuch as I have wished to name genetic factors rather than species of cats, I have discarded the Latin names and have adopted the English words blotched and striped, in regard to which there can be no confusion.

⁴ By the use of the term 'mimic' I wish merely to denote what in my estimation underlies many at least of the phenomena which biologists have attempted to explain by the mimicry hypothesis. There are only a limited number of ways in which an organism may vary. Thus a mammalian coat may vary in dilution and distribution of the pigments black, brown, and yellow. No other pigments can be developed.

Numerous cases of resemblance, moreover, are in all probability due to homologous factorial differences, even in widely separated species. Metz ('16)

On the other hand, variations in the ticking factors and in the banding or pattern factors occur both in wild and in domestic mammals. Such variations in the domestic cat produce color patterns closely similar to numerous wild species. Variations from red to silver occur in wild cats. The tiger has a high degree of red with a moderate amount of ticking. Thus the pattern is very well marked. In the lion and the puma as well as in the jungle cat and others, the red is reduced to yellow while the ticking is very intense. Hence the pattern appears only in young animals and is obliterated by the increase of ticking incident with maturity. Other cats like the ounce or snow-leopard and Pallas' cat represent an extreme reduction of yellow pigment comparable with silvering in domestic tabbies. Loss of agouti producing black varieties of leopards and others are well known. Small species of African and Asiatic cats vary so in color that much confusion has resulted in taxonomy. All of this diversity may apparently be reduced to variations in ticking, in banding, and in the red-silver series. Spots, I believe, are produced by crossing of longitudinal and transverse waves of pigment-forming metabolic activity. In these respects the domestic cat tends to 'mimic' its wild relatives, but whether the variations have originated by crossing or by mutation is an open question.

IV. THE SUMMARY

The inheritance of color variations in the domestic cat has been investigated at the Zoological Laboratory of the University of Pennsylvania.

Maltese dilution segregates distinctly from intense color and is probably recessive.

Solid white is a simple and complete dominant over color. White-spotting is very irregular in inheritance. There is partial correlation between dominant white, blue eyes, and deafness.

has shown that mutations have occurred in *Drosophila virilis* (species B) producing characters similar to mutant characters in *D. ampelophila*. Such variations are inherited according to a similar mechanism and show comparable linkage relationships. I am of the opinion that resemblances in the colors and patterns of different mammals are often due to such genetic homology.

It is suggested that irregularities in inheritance of blue eyes and deafness may be explained by correlation with white-spotting.

Yellow is determined by a sex-linked factorial difference from other colors. The heterozygous female is usually yellow-spotted or tortoiseshell, but ranges to solid black or tabby and to solid yellow. It is suggested that yellow-spotting in the male depends upon extreme selection or segregation of other factors.

Kittens resembling adult Siamese cats have been produced from common cats.

Banding cleanly segregates in three different widths. It is probable that the factorial differences involved act as triple allelomorphs.

Much-ticking, little-ticking, and black probably constitute a triple allelomorphic series. Intergrades occur between much-ticking and little-ticking.

Ticking follows banding in its distribution.

Black-spotting in wild Felidae and in domestic tabbies is explained by crossing of transverse and longitudinal bands.

Color varieties are classified as those that 'mimic' other domestic animals, and those that 'mimic' wild species.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

A. Skin of silver-striped tabby, probably little-ticked, but with reduction of pigment due to silvering.

B. Skin of much-ticked striped tabby. The longitudinal bands are obvious as well as the tendency of the transverse bands to become broken into spots.

C. Skin of little-ticked lined cat.

A and C are from sibs from cross of dark-ticked lined by black.

D. Skin of little-ticked striped cat.

B and D are from sibs.



PLATE 2

EXPLANATION OF FIGURES

E. Skin of much-ticked lined kitten at birth.

F. Skin of much-ticked blotched kitten at birth.

E and F are from sibs from cross of little-ticked lined by much-ticked blotched.

G. Skin of little-ticked lined kitten one week old.

H. Skin of little-ticked blotched kitten one week old.

G and H are from sibs from cross of little-ticked lined by much-ticked lined.

The animals, the skins of which are shown in figures A, C, E, F, G, and H, had the same male parent, a little-ticked lined or Caffre cat owned by the Zoological Society of Philadelphia.



THE INFLUENCE OF EXCESSIVE SEXUAL ACTIVITY
OF MALE RABBITS

II. ON THE NATURE OF THEIR OFFSPRING

FRANK A. HAYS

Iowa State College

TWENTY-TWO CHARTS

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INTRODUCTION

Too frequent copulation of males is often given as an important cause of weak and inferior offspring from the standpoint of growth and thriftiness. This idea seems to be rather universal, though evidence of such being the case is difficult to obtain.

Wright (p. 306) states that in his poultry-breeding operations he does not expect normal size or vigor in offspring from cocks used on too many hens, and further (p. 131), that cocks that are used on too many hens show the effect in that the eggs fertilized by them show signs of hatching but do not hatch because the embryos fail in many cases to reach full development. Pusch ('15, p. 182) expresses the almost universal belief in this matter, though he does not consider the idea well grounded when he writes: "Braucht man die Deckhengste, wie überhaupt jedes männliche Zuchtthier, zu stark, so schädigt man nicht nur deren Begattungs- und Befruchtungsvermögen, sondern auch die Qualität ihrer Nachzucht; deshalb wird die Stutenzahl für wertvolle Vollbluthengste auch nur auf 30-40 Stück bemessen und die zu bedeckende Stute erst durch den Probierhengst auf ihre Rassigkeit hin geprüft." Day ('13, p. 219) expresses the belief that excessive use of the boar is likely to result in small, weak litters of pigs.

Just why sperm cells that are produced by a male in heavy sexual service should produce inferior offspring when they take part in fertilization is not clear. Can it be possible that the genetic makeup of the spermatozoa is changed by heavy service? Is it not possible that any sperm cell possessing life, however depleted and weak it may be, will carry into the egg a potentiality of full vigor? Or, on the other hand, can we conceive of different degrees of vital force in a sperm cell? Since all of the activities of the animal body are so beautifully coördinated, it would appear very rash to assume without conclusive evidence that males under natural breeding conditions would derange any vital function, such as reproduction, by continuing to copulate after the reproductive system was producing an abnormal product. As has been pointed out in our first paper (Lloyd-Jones and Hays, '17), there appears to be a relation between the number of services performed by the male and the fertilizing power of his semen, but as far as we have been able to measure, we are led to believe that only a slight change can be brought about by this treatment. Pusch ('15, p. 182) states that in Oldenburg, stallions are often allowed to make from four to six or even eight

services daily. He also states that bulls have been used on 400 cows in a year, and that even poorly fed bulls will make from four to eight copulations daily, and this without bad effect.

Strictly speaking, 'vitality' of individuals cannot be measured, for the vitality of any individual really means the sum total of life force within every living cell of the organism; vitality, as used in speaking of animals, may, however, in part be measured by the rate of growth in weight, the skeletal development, and the ability of the individual to live to a good age. Such factors as body weight and the others mentioned above are measurable. The purpose of this investigation has been to study the effects of heavy service of males on the nature of their offspring, as far as we could measure the effect.

MATERIAL AND METHODS

1. Animals used

The character of the animals used in this investigation has been discussed to some extent in the first paper of this series. Stocks of the European domestic rabbit, *Lepus cuniculus*, secured from six different breeders, were used and no inbreeding was practiced at any time. The weight¹ and age of the females is an important factor in that it affects both the number in a litter and the individual weights of the offspring. Likewise the weight of the male probably is of much importance in affecting the weight of the young. The maturity of the male is a factor that should not be lost sight of, because all three of the males used were fully mature and were in their prime of life—about two years old. The average weights of the males are as follows: No. 1, 2850 grams; No. 3, 2575 grams; and No. 4, 2200 grams.

Shy breeders sometimes occur in rabbits, but most of these females proved to be regular breeders. No. 25, however, was barren and was discarded; No. 12 produced young three times, the last time August 5, 1916, after which time she appeared never to come in heat again and continually refused to copulate

¹ Prof. H. W. Vaughan has found that Large Type Poland-China Swine produce larger litters than the Small Type.

and was discarded; both No. 22 and No. 29 died after having given birth to but one litter upon which we secured data; and No. 18 died after she had given three litters to the experiment.

Age of the dam is an important factor as affecting the number in the litter and probably to some extent the weight of the individuals of the litter. For this reason the approximate ages of the breeding animals is here given in order that the reader may understand fully how much error may have been introduced through immaturity in the breeding females. One female gave birth to young when six months old; two, when seven; four, when nine; one, at ten, and one, at eleven months old. The remainder of the females was fully mature, that is, fifteen months old or over, at the time they gave birth to the first litter used in this experiment.

The fact should be noted that the three females that died during the experiment were all immature at the time they first reproduced and that only one of them (No. 18) had more than one litter upon which we secured data. Two of the three litters from No. 18 are 5th-service litters by male No. 3 and the other is a 1st-service litter by the same male. Female No. 22 gave a single litter from the 5th-service by male No. 1; and female No. 29, one litter from the 1st-service by male No. 4; female No. 12 has contributed but two litters to the records; namely a 1st- and a 10th-service litter by male No. 1.

It may appear to the reader that considerable error, resulting from the use of these immature females, was overlooked in making up our records of growth, but this has not been the case; therefore, a brief consideration of the system of matings used to overcome this error is not out of place here.

The system of matings was arranged so that each female was mated to at least two of the males and many to all three males, and where possible each female produced litters from all different services from each male, thus reducing parental variability to the males alone. By making the three breeding groups of females as nearly equal as possible in age and weight; by distributing the heavy service among the females in such a way as to secure all types of litters from both mature and immature fe-

males, and by making matings at such a time as to secure all types of litters at the same season of the year, as far as possible, we hoped to overcome many possible sources of error. However, as the experiment proceeded it was found impossible to apply these corrections absolutely and we are thus not fully justified in comparing litters in growth in body weight and in mean dimension and assuming that any consistent differences are due to the number of services performed by the males. We should not overlook the slightly better opportunities offered the 15th- and 20th-service litters, a considerable proportion of which were born during the latter part of the experiment and were produced when the females were all mature.

2. Records kept

The following records were kept: Date of breeding, pedigree, date of the next probable heat period—fifteen days after breeding; actual date of parturition; number in litter; number born dead; sex of offspring; individual weight of offspring on day of birth and for each five days thereafter up to ninety days; head length and breadth through extremes of ilium, taken at the same time as the weights; date of weaning; color, and mortality record.

3. Weighing and measuring

Breeding records were kept for each female so that it was possible to weigh each litter on the actual day of birth. At this time each litter was given a number which was the same as the number of the matings, and each individual was given an individual number and marked in such a way as to be easily distinguished from litter mates by color description or by clipping ears and tail. The individual weight records were kept each five days until the litter reached the age of ninety days.

The desirability of continuing all records to the full maturity of the progeny is very apparent. As the work was handled, forty or fifty animals were often weighed and measured on a single day and, with the other routine work of the experiment, entailed a

very large amount of labor. Such extensive records were impossible for reasons that need not be discussed here. Weights were secured on a sensitive torsion balance and variations of 0.5 gram were recorded. Great errors may be introduced by a 'fill' if the records are not made at the proper times; therefore the records were secured at about the same hour each day before feeding, which was done once daily. However, there are certain errors in weight records which cannot be avoided by the experimenter. The general degree of health of the animals has much to do with fluctuations in weight as MacDowell ('14) found in growth studies of rabbits; nevertheless, as with other animals, weight seems to be the best available index of growth.

Two methods for studying the growth of the progeny produced were chosen, namely, growth in body weight and growth in body measurements. The first will be discussed here.

Body weight, according to Minot ('08, p. 87), represents the total mass of the living body, while body measurements are only partial indices of growth. That individuals show wide fluctuations in weight has been pointed out by MacDowell ('14, p. 191) in his studies on the rabbit. Although growing rabbits show marked variability in weight on different days, it was thought possible by the use of large numbers to secure growth curves that would fairly represent a race of rabbits kept under uniform conditions. There is a possibility that these growth curves would diverge more as the animals grow older, because MacDowell has shown that though most rabbits apparently make a normal growth to maturity, others fall much below the normal and do not reach the average weight in what is considered the normal period. But complete records were out of the question as indicated above. Even though this is the case, it is very important to ascertain if this reputed inferiority of progeny which is supposed to result from the weaker sperm cells of the overworked male is going to be apparent when his progeny are in the most active stage of growth, i.e., during the first ninety days of postnatal life. If progeny from the advanced services of males are more poorly equipped with the necessary something to enable them to make normal growth, would this not be apparent

when the young rabbits are thrown upon their own resources, as was done during the sixty days following weaning time that the records were kept?

Concerning the second method of studying growth, namely, by body measurements, it is important to discover whether body development follows apace with body weight and to check one against the other. For this reason, all litters born up to August 9, 1916 (45 in number), were measured as well as weighed, at five-day intervals up to the age of ninety days.

A measure of head length was considered valuable, as measurements of the skull have been found to be less variable than measurements of long bones. MacDowell ('14, p. 38) found this to be true in rabbits. Hatai ('08) observed the same thing in the albino rat, and Quetelet ('71) likewise found the same in man.

The head length as here reported was measured by the use of calipers and represents the distance obtained by placing the stationary arm at the crest of the occipital bone allowing the beam of the calipers to extend sagittally downward parallel with the face. The movable arm was then brought up until it rested snugly against the end of the nose and down over the mouth. The lower arm of the instrument was then just beneath the inferior extremity of the premaxillae and the upper arm was just above the superior region of the occipital bone. There is very little flesh or soft tissue covering the bones in this region, about the only structures obscuring the bones are the skin and the hair coat. It is apparent, that a head measurement in this particular region approximates rather closely the actual skull size.

Since there is the possibility that some other body measurement would make an entirely different growth curve from that of the above-described head measurement, it was considered desirable to secure one other measurement that could be taken with considerable accuracy on the live animals. Moreover, we wished to obtain a 'mean dimension' from the average of two measurements, therefore some easy body measurement was searched for. There is no little difficulty in securing external measurements of the body with accuracy, as the writer has learned from much experience with cattle and swine. A measure of the breadth be-

tween the extremes of the ilia was thought to be as easily determined as any of the possible body measurements and would represent a dimension of breadth in contrast to head length, which might be considered a dimension of depth. The iliac expanse was therefore used as the second measurement.

Both measurements were taken just after weighing on the five-day periods beginning at birth and continuing to the age of ninety days. Steel calipers were used with vernier graduated to hundredths of a centimeter. Three independent readings of each dimension were taken by removing the calipers and shifting the arm after each reading. Readings were put down just as read, and care was taken to avoid any tendency on the part of the observer to modify readings to make them check with others. As a rule, it was possible to obtain readings that varied less than 0.1 centimeter from each other. An average of the three readings was taken as the correct reading for each measurement.

Little difficulty was experienced in securing what was considered a correct reading on head length. This was not always true for the other dimension. Three factors probably enter to modify this reading: 1, the amount of 'fill;' 2, the degree of fatness; 3, the position of the hind limbs. Food and water in the alimentary tract seem to bulge the walls of the abdomen to such an extent as to often obscure the points of the ilium and make their exact location difficult. The variability of the feeding habits of the rabbit is thus a factor of no little importance in connection with the measurement of iliac extremes. Some individuals carry much more fat over the ilium bones than others. In fat individuals the points of the bones are often greatly obscured, especially in the younger rabbits. This condition is much more common among the smaller and better nourished individuals and may prevail to some extent throughout the period of observation. There is considerable flexibility in the pelvic girdle before the symphysis pelvis becomes bony and firm as the animals approach maturity. The ilium, ischium, and pubis are also distinct and more or less flexible in early life. This great flexibility causes the position of the hind limbs to be an important factor in modifying the position and the breadth of the ex-

tremes of the ilium as determined by the calipers. In so far as possible an effort was made to have the animals sit with the limbs in the natural position while being measured. The hair was also clipped from this region of the body in order that it might not obscure the point of the bones.

4. Methods of interpreting weights and measurements

In order to make the data for different-sized litters more nearly comparable, all weight and measurement records are reduced to an 'individual mean' for each litter for each of the nineteen periods of observation. The individual mean for each litter was calculated by dividing the total weight or total measurements of each litter by the number of individuals for each of the nineteen periods. From these individual litter means the series of cumulative growth graphs are constructed.

In attempting to compare the growth graphs of rabbits in the different service groups, a very perplexing problem arose as to how to best compare results in litters that vary so much in number of individuals. The number of individuals born in a litter is an intensely important factor in influencing the weight of the young. Our observations have shown this as did also observations of Minot ('91, p. 111) on guinea-pigs. His results, based upon 351 observations, show that the average birth weight is 85.5 grams in litters of one, the weight gradually decreased with the increase in number of individuals to as low as 52.2 grams in litters of eight.

Another item that makes comparisons of litters in different service groups difficult is the fact that litters in the 1st and 5th service groups are likely to contain more individuals than those from the 15th and 20th services. For this reason the individual mean of these advanced service litters is greater and they grew faster because of a more generous supply of milk from the mother. In this connection we find that King ('16, p. 51) discovered that in rats "body weight at birth indicates the probable capacity of the individual for subsequent growth." This being the case, small litters from the advanced service should grow more rapidly than the larger litters from the 1st and 5th services.

In order to make the litters in the different service groups comparable with each other, whatever their number, it was thought first that litters of different numbers of individuals could be standardized to a mean litter number. Jackson ('13, p. 17) in comparing the standard deviation of individual rate with the standard deviation of the entire race, reduced all individuals to a common basis by multiplying the body weight of each rat by a factor obtained by dividing the mean of the total population at a given age by the mean of the given litter. Since the object we have in view is not the study of individuals, this formula cannot be used. Further attempts were made to obtain a factor for reducing large and small litters to a comparable basis, but so far with no success. Again, it was thought possible that the coefficient of correlation between number of individuals in the litter and average weight per individual might be made use of to reduce the litters to a comparable basis, but without any satisfactory results. Again, a comparison of different-sized litters in the several service groups by constructing graphs upon a base line representing the different litter numbers and the vertical line representing the variable weights at birth, a second chart to show the time required to make eight times the birth weight, and a third chart to show the time required to make twenty-four times the birth weight were attempted. By this means the data could be much condensed, but such a system proved to be impracticable and was discarded. Finally, it was deemed best to compare litters of the same number of individuals. Accordingly, the growth rate in the different service groups must be shown by a whole series of charts, the graphs on each chart representing a certain litter number. In each case the chart shows the number of litters which are lumped in each graph. Thus each chart gives a direct comparison of the growth rate of the five service groups, namely, 1st, 5th, 10th, 15th, and 20th, the comparison being always between litters of the same number.

As a further measure of divergence in rate of growth between the service groups, the coefficient of variability of weight for all litters in each of the five service groups is valuable, presented

at birth, at weaning time or thirty days, and at ninety days. The object here sought is to find out if there is a greater variability in any one of the service groups, which might be expected if any of the service groups contain weak offspring. This study will also reveal if heavy service tends to produce a wide range of variability in birth weight or a wide range in the weights of individuals at the time that they are thrown upon their own resources at weaning time, and it will show further if the individuals tend to deviate more from the mean as they grow older. Deviations, if they are going to occur, might be expected to occur, more strikingly at these three periods than at any other time during the observations. This coefficient of variability was determined by the following formula:

$$\text{For each litter in service group.} \left\{ \frac{(\text{Sum of deviations of litters from mean})^2 \times (\text{frequency of class})}{\frac{\text{Number of individuals.}}{\text{Mean of respective litters.}} \times 100} \right\} \left\{ \frac{\text{Number of litters in service group.}}{\text{Number of litters in service group.}} \right\}$$

The above formula is used for the birth weights, the thirty-day weights, and the ninety-day weights.

The measurement data secured were combined into one general expression, the 'mean dimension.' The advantage of using one expression to stand for body measurements lies in the fact that we have a mathematical expression for the cross section of the animal. Graphs expressing cumulatively the percentage increase in head length and iliac extremes are found to cross between the thirty-fifth and fortieth day of postnatal development; but previous to this date and later, up to the time of the conclusion of the observations at ninety days, the graphs bear a close relation to each other, therefore it was deemed correct to combine the two measurements to obtain the mean dimension.

The mean dimension was obtained by the following formula:

$$\frac{\text{Mean head length} + \text{mean iliac extremes.}}{2}$$

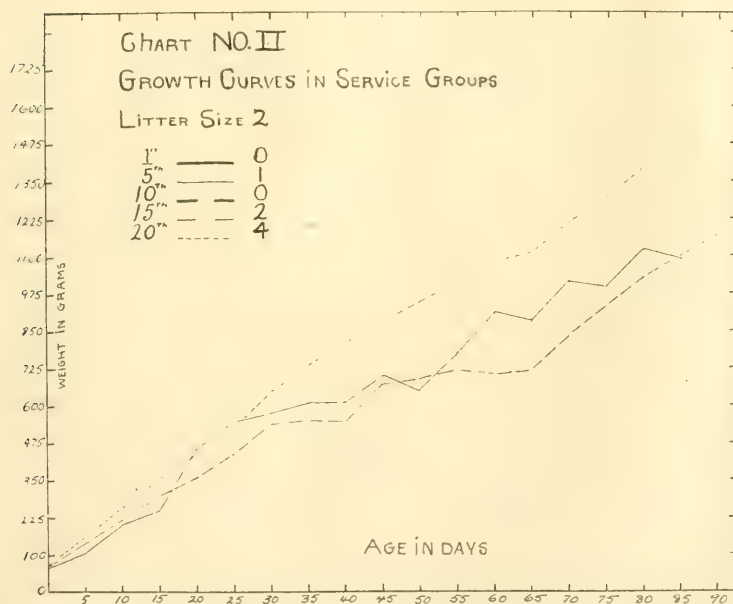
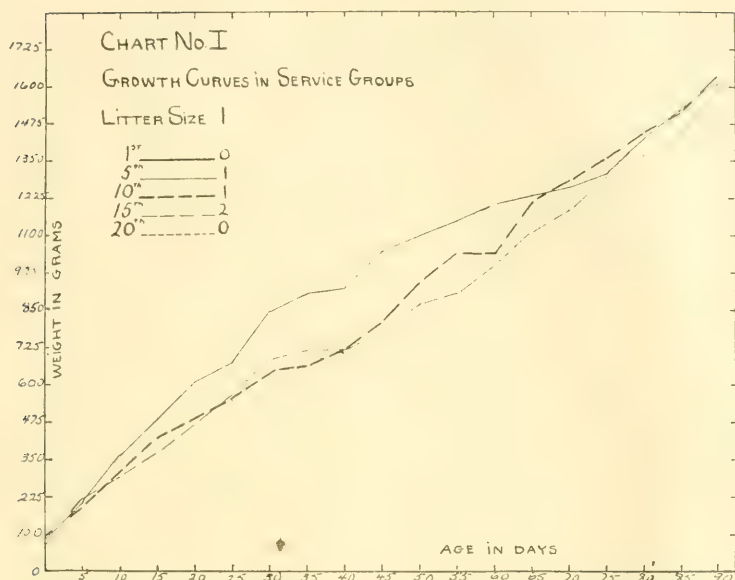
Mean head length is the sum of the averages of the three readings for each individual in a litter divided by the number of individuals in the litter. Mean iliac extremes represents the sum of the average of the three measurements divided by the number in litter. By using the above formula for mean dimension, the calculation was made for each litter for each of the nineteen periods of observation. Graphs presented on the measurement data are made up in exactly the same way as has been described for making the graphs for weight, comparing only litters of the same number of individuals. A grand average graph is likewise made up regardless of litter size. The graphs based upon identical litter size are considered reliable for purposes of comparing the offspring in the different service groups, but the grand average graph is subject to considerable error.

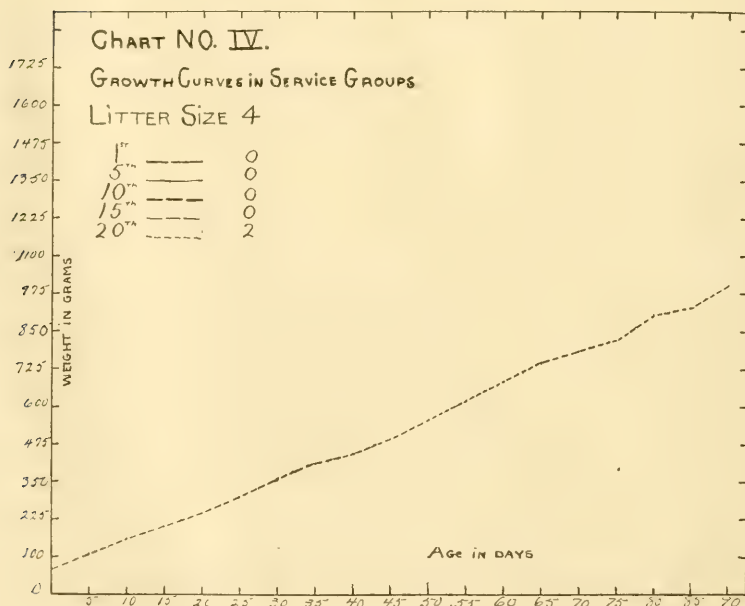
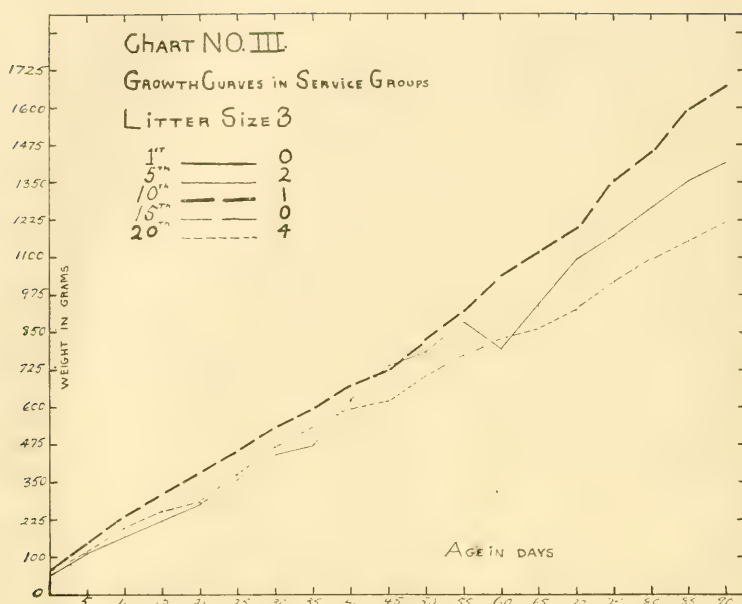
DATA AND RESULTS

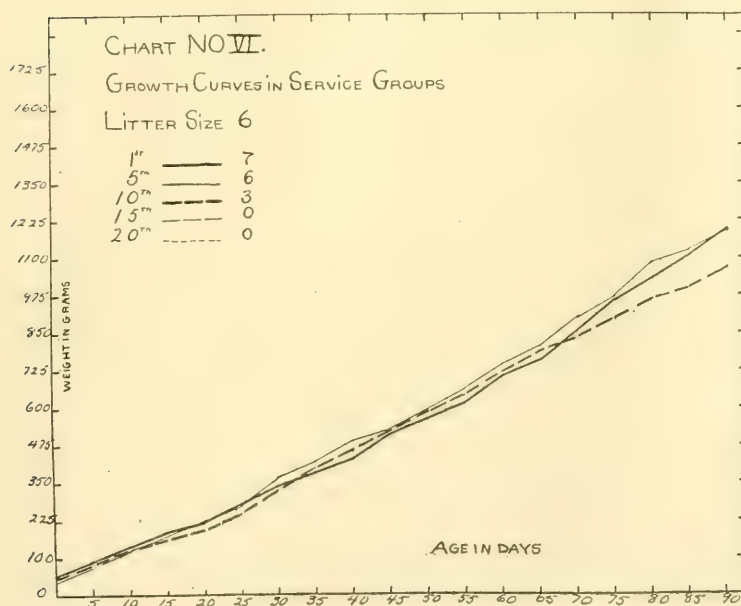
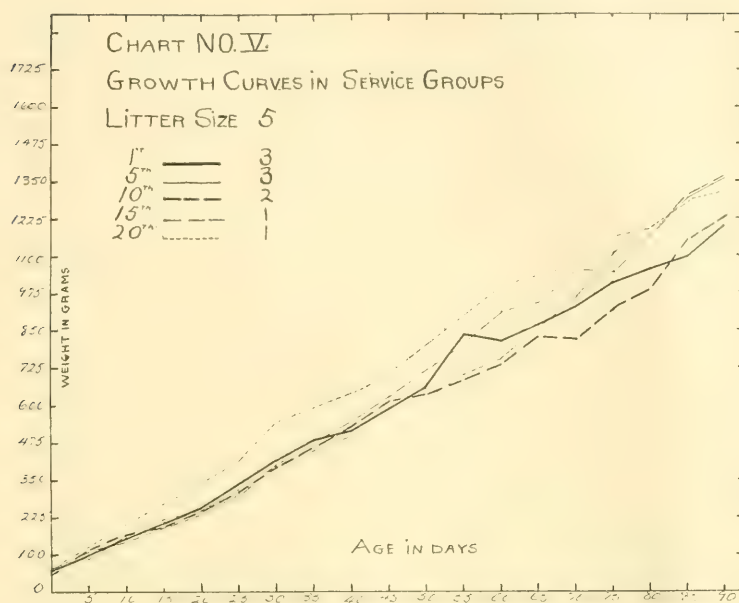
1. Growth in weight of young as related to frequency of copulation of sire

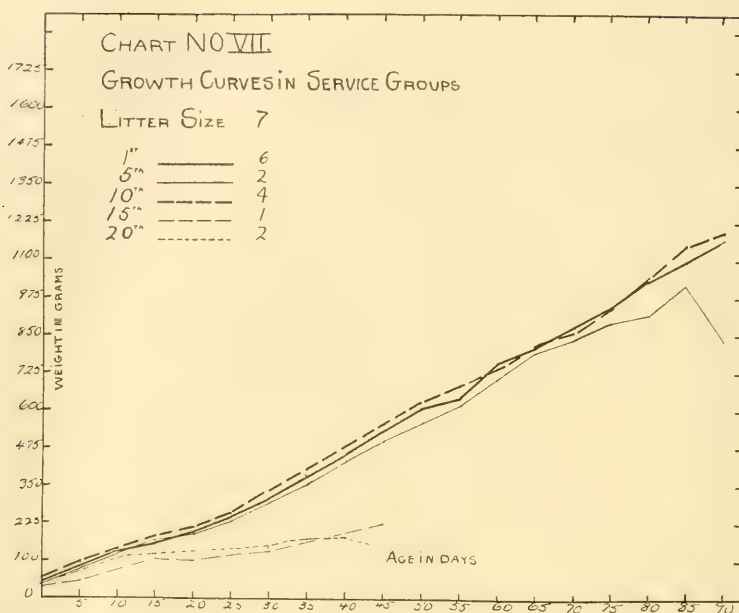
For studying the offspring with a view of determining if there is any relation between the number of services made by the males and the rate of growth, there appears to be no better measure than body weight. Body weight measures the animals as a whole and should thus reveal any inherent weakness that retards their growth.

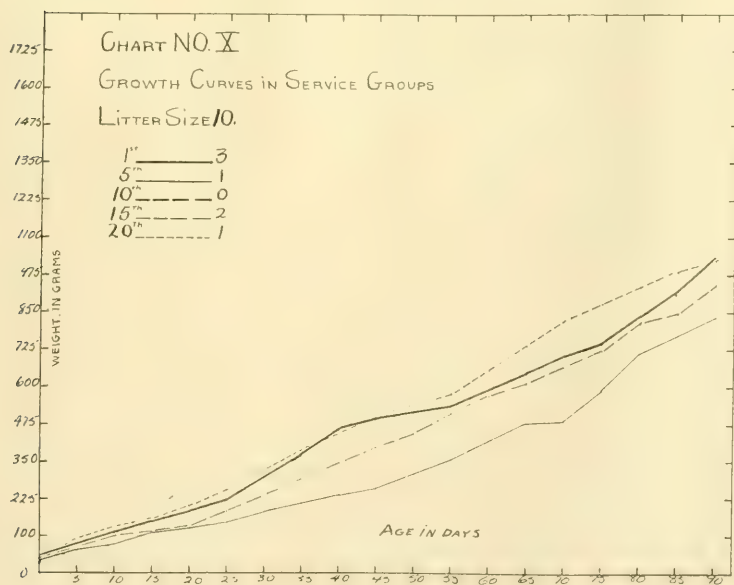
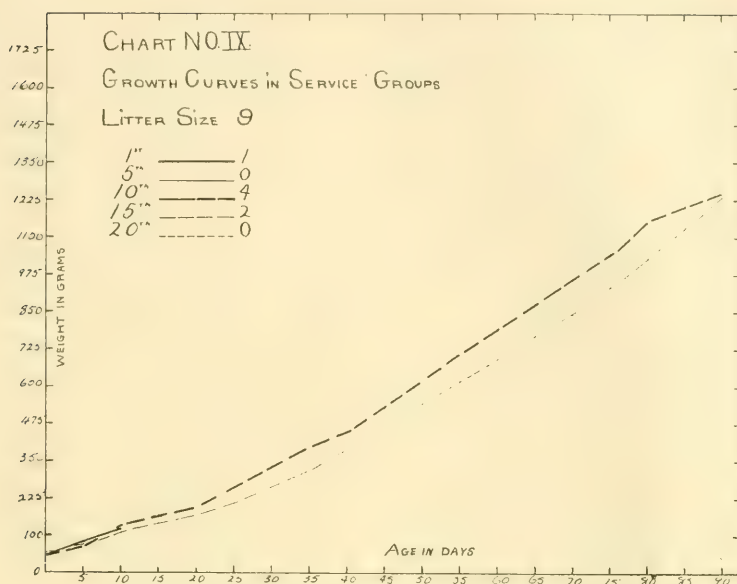
Below are presented charts 1 to 12 taken from the composite weight records of the young of all three males. Each chart represents a single litter number at birth, all litters of one size, in each of the five service groups being grouped together and the same grouping being followed for all litter numbers as described on page 580. Each service group is represented by a different line, as is disclosed by the legend on the charts. The number of litters represented by each curve is given in each case. Chart 13 shows the weighed grand average for all five service groups. It was obtained by adding together the individual mean of each litter and dividing by the total number of litters at each observation period. We have already shown that such a chart comparing directly the growth ratio of the different service groups

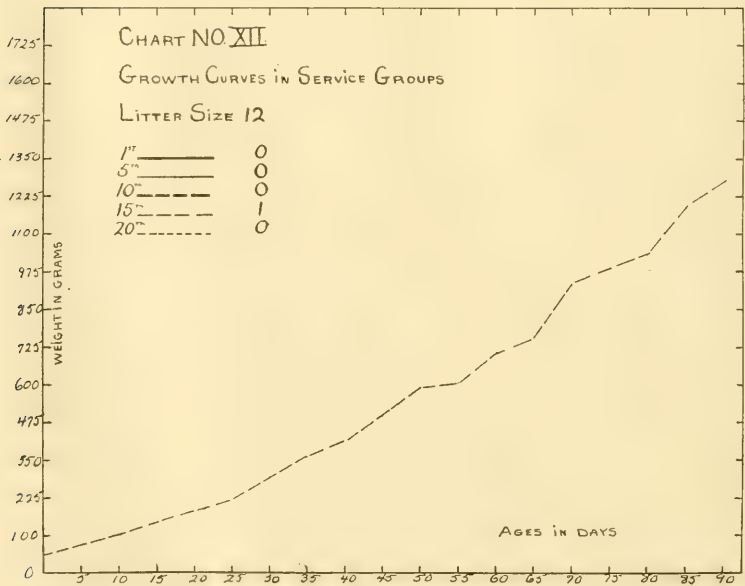
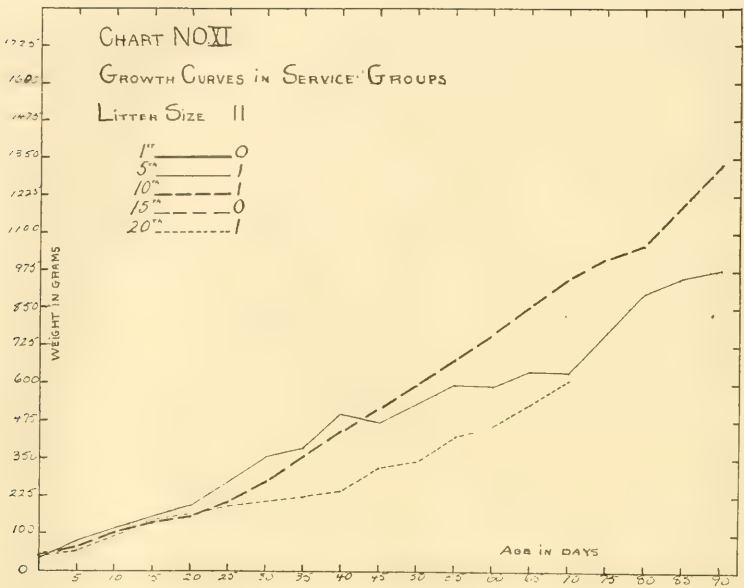


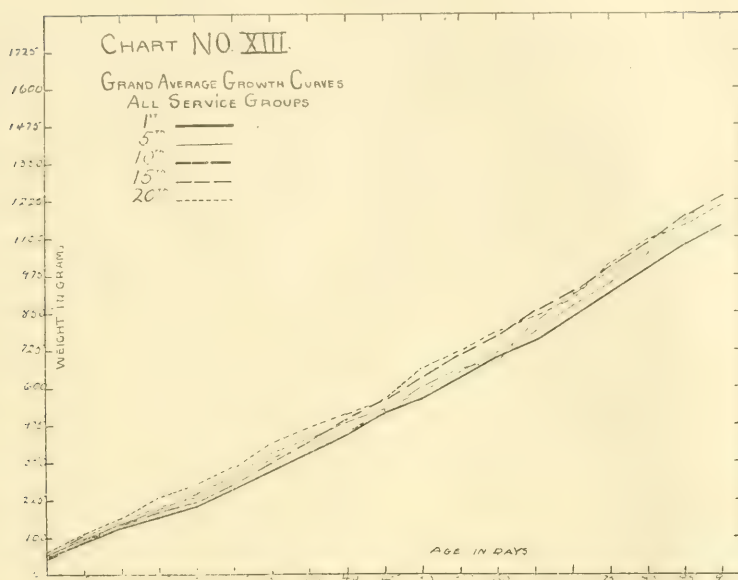












may not be made with absolute justice because, as will be shown later, the litters in the advanced service groups tend to be smaller. A rough comparison of all progeny may be made in this way, however.

Charts 1 to 12, inclusive, present the results in a form that may be easily grasped by the reader, but there are a few points revealed by a study of these graphs that require some discussion. With but few exceptions, the 20th-service graph lies above all other graphs. This is a striking and surprising result and the question at once arises as to the cause of the almost uniform heavier birth weight and more rapid growth of the 15th- and 20th-service litters compared with litters of the same size from less advanced services. The results are in direct contrast to what, according to the traditions of breeders, would be expected. On their face they actually show that the heavier the service of the male, the more thrifty the offspring. It seems best to here consider the possible factors that may play a part in causing the superiority of these advanced service litters over litters from the 1st, 5th, and 10th service.

During the production of the majority of the 1st- and 5th-service litters the breeding animals were housed in somewhat cramped quarters. Conditions there were not conducive to the most rapid growth of the young and were not as favorable for the breeding females because of small space and rather poor ventilation and poor light. Furthermore, the progeny were crowded into rather limited exercising pens, and probably for this reason they did not develop at so rapid a rate as would have been the case under the more favorable quarters used later. The majority of the 10th-service litters, on the other hand, and about half of the 15th-service litters were produced while the stock was housed in more ample quarters where the space was large, the ventilation good, and everything was conducive to health and thriftiness. In fact, the quarters used at that time were practically as good as the present permanent and excellent quarters where the 20th-service litters were produced. The superior environment of the advanced service litters is no doubt partly responsible for the greater growth of the advanced service litters compared with the moderate service litters; but environment cannot be entirely the cause of the superiority of the 20th-service litters over the 15th, and the 15th-service litters over the 10th-service litters. Let us therefore seek a further explanation.

Parentage may be an important factor affecting the weight. As has been previously noted, the variability of the female breeding stock is considerable, the range of weight was from 2500 to 3250 grams, averaging 3050 grams, but the females have been so distributed among the three breeding males as to make three groups of practically uniform weight and variability in size. Nevertheless, lack of uniform weights in the progeny may still be partly due to variability of the female breeding stock.

The size of the sire may also be a factor in controlling individual mean weight. The three sires used were quite different in weight; their weights are as follows: No. 1, 2850 grams; No. 3, 2575 grams, and No. 4, 2225 grams in ordinary breeding condition. Male No. 1 sired eleven of the seventeen litters included in the 20th-service group. He, being the largest of the three males, would be expected to sire the heaviest offspring at birth, and such offspring

TABLE 1

Average birth weight of litters sired by the three different males used, by service groups

| MALE MEMBER | 1ST | | 5TH | | 10TH | | 15TH | | 20TH | |
|-------------|---------|--------|---------|--------|---------|--------|---------|--------|---------|--------|
| | Num-ber | Weight | Num-ber | Weight | Num-ber | Weight | Num-ber | Weight | Num-ber | Weight |
| 1 | 6 | 46.7 | 6 | 45.2 | 7 | 45.2 | 7 | 52.5 | 11 | 59.7 |
| 3 | 12 | 50.9 | 8 | 45.0 | 2 | 45.0 | 2 | 42.7 | 00 | 00 |
| 4 | 8 | 42.4 | 6 | 58.8 | 4 | 58.8 | 4 | 49.6 | 6 | 53.3 |

Weight at ninety days

| | | | | | | | | | | |
|---|----|--------|---|--------|---|--------|---|--------|----|--------|
| 1 | 5 | 1209.5 | 3 | 1236.6 | 7 | 1238.1 | 7 | 1262.0 | 6 | 1299.3 |
| 3 | 11 | 1170.0 | 6 | 1217.8 | 7 | 1095.3 | 2 | 1270.7 | 00 | |
| 4 | 7 | 1054.9 | 6 | 1096.1 | 5 | 1424.3 | 2 | 1199.7 | 3 | 1009.0 |

could be expected to keep ahead of the other classes of offspring at least for ninety days. This way of explaining the position of the 20th-service graph above the others is called in questions by chart 3 and also by table 1. The graph of the 20th-service litter lies below the others. This graph represents the growth of a single 20th-service litter (after the first weight) also by Male No. 1 and out of the heaviest female in the breeding stock (No. 15). Therefore, the fact that this litter lies below 5th- and 10th-service litters on this chart cannot be explained as the result of small ancestry.

Table 1 shows that the size of the male ancestor is not a very important factor in relation to the size of the young at birth. At the age of ninety days, however, the effect of the heavier sire becomes more important, but nevertheless is probably not as important as some other factors concerned as will be pointed out later.

When we consider the 15th-service group, we find that seven litters were sired by Male No. 1, two by No. 3, and four by No. 4. Again we should expect a more uniformly heavy progeny than if all males had contributed an equal number of litters to the data. Chart 8 shows the superiority of the 10th-service group over the 15th-service group up to the fifty-fifth day, after which time the graph rises above all others.

Thus far we have attempted to account for the heavier weights and the greater rate of growth of the advanced service litters as due entirely to factors other than the nature of the spermatozoa and not to any inherited superiority. The effect of such factors does not seem adequate to explain the apparent superiority of the advanced service litters, therefore there is good evidence that a real superiority exists among the advanced service litters as compared with the light service litters. The female ancestors in both service groups were practically equal in weight. One of the 15th-service litters represented in chart 9 was sired by Male No. 1, the other by No. 3. Two of the four litters combined in the 10th-service graph were sired by No. 1 and two by No. 3. The smaller weights of the 15th-service litters during the early part of the observations cannot for the above reasons be explained by male ancestry of different weights.

One other hypothesis may be proposed to account for the probable superiority of the advanced service progeny over those from very moderate service. Pearl ('17, p. 296) treated both cocks and hens with ethyl alcohol, methyl alcohol, and ether at different times during the breeding season in order to study the effects on their progeny. He found the offspring from treated parents in every way superior to those from untreated parents. Pearl assumes that alcohol and other poisons act as selective agents upon the germ cells of treated animals. It is possible that selective action might be brought about by heavy sexual service of the male. We have previously shown that heavy sexual service induces the liberation of sperm which often show no progressive motion and are short-lived. Some few of the sperm from these advanced services do exhibit the physical properties that indicate high vital force. The possibility exists then that what few spermatozoa do take part in fertilization are superior to the average in the light service groups because the bulk of the spermatozoa in the advanced service groups are not equipped to take part in fertilization, while this is probably not true in the light service groups. Such a hypothesis as the above will thus account for the superiority of the advanced service progeny.

TABLE 2

Number of litters included in graphs of charts 1 to 12, inclusive, and the male ancestry

| MALE MEMBER | SERVICE GROUP | | | | |
|----------------|---------------|-----|------|------|------|
| | 1st | 5th | 10th | 15th | 20th |
| 1 | 6 | 6 | 7 | 7 | 11 |
| 3 | 12 | 8 | 7 | 2 | 0 |
| 4 | 8 | 6 | 6 | 4 | 6 |

Concerning the graphs for the 10th-, 5th-, and 1st-service litters, we note that as a rule the 1st-service litters are inferior in weight to either the 5th- or 10th-service litters and that the 10th-service litters are for the most part superior to the 5th-service litters. As previously noted, less favorable environment and greater immaturity of some of the female animals are thought to be the chief factors entering here. The male ancestry is almost uniformly distributed among the three males. Below we note from the table just how the ancestry is distributed.

Table 2 shows us that the three males are about equally distributed in the progeny groups from the 5th and 10th services. In the 1st-service group, however, No. 3 has sired twice as many litters as No. 1 and 50 per cent more than No. 4. Since Male No. 3 is a smaller animal than No. 1, we have here a partial explanation for the apparent inferiority of the 1st-service litters over all others. In the 15th- and 20th-service groups the progeny of Male No. 1 predominate, and Male No. 3 sired no litters in the 20th-service group.

A word of explanation in regard to a few remarkable features of some of the charts may be of value at this point. On chart 3 the depression in the 5th-service graph at sixty days is due to a failure to obtain data on the heavier of the two litters making this graph. This particular litter was unintentionally overlooked for four weighings. On chart 5, the drop in the 10th-service graph at sixty-five days is due to the incomplete record on one litter at the time the graphs were constructed and this litter was made up of very heavy individuals.

Chart 13 represents the grand average growth of all litters in the five service groups as explained on page 582. Each graph thus represents the individual mean for the combined litters in each service group. These composite service group graphs bear out the general deductions that we have made from a study of the graphs taken one by one comparing litters of a given number with each other in the five service groups. There is one outstanding objection to the use of such graphs as are shown on chart 13. There is a perceptible negative correlation between number of services of the sire and the number of offspring in litters resulting (Lloyd-Jones and Hays, p. 492). In other words, heavy service does reduce the size of litters, especially in the two most advanced service groups used here. Consequently the greater supply of nutrients furnished by the mother in utero as well as the greater supply of milk available after birth will enable the advanced service litters to outstrip the other litters during the periods of observation in this experiment. This condition would hold if all litters were equally fit genetically; and we have no evidence that any class of offspring is rendered less fit by heavy service of their sire.

To recapitulate, certain errors have been introduced into the growth studies in body weight, chief among which are environmental factors, the age and weight of the dam and the weight of the sire. These errors have been partially corrected, and the conclusion seems justified that there is no evidence in this data to show that the amount of sexual service that the male has been required to perform in any way affects the rate of growth of his offspring in body weight for the first ninety days of postnatal life.

2. Litter coefficient of variability

The coefficients of variability in table 3 presented below were obtained in the following manner: The coefficient of variability for each litter in each of the five service groups was determined at birth, at thirty days, and at ninety days by the formula:

$$\frac{\text{Standard deviation of each litter.}}{\text{Mean of the litter.}}$$

TABLE 3

2. Coefficient of variability of individuals within the litters at three different periods

| AGE | SERVICE | | | | | | | | | |
|-------------|-------------------|------------|-------------------|------------|-------------------|------------|-------------------|------------|-------------------|-----------|
| | 1st | | 5th | | 10th | | 15th | | 20th | |
| | Number of litters | Per cent | Number of litters | Per cent | Number of litters | Per cent | Number of litters | Per cent | Number of litters | Per cent |
| <i>days</i> | | | | | | | | | | |
| Birth | 26 | 10.81±1.01 | 20 | 10.73±1.14 | 19 | 12.52±1.37 | 11 | 11.43±1.64 | 16 | 8.80±1.05 |
| 30 | 23 | 10.72±1.07 | 17 | 8.27±0.96 | 19 | 10.05±1.10 | 11 | 9.56±1.29 | 11 | 7.89±1.11 |
| 90 | 23 | 10.10±1.02 | 14 | 6.77±0.86 | 17 | 7.55±0.87 | 8 | 10.70±1.80 | 9 | 8.94±1.42 |
| Average. | | 10.55 | | 8.82 | | 10.13 | | 10.55 | | 8.56 |

The coefficients of variability for all 1st-service litters at birth were then added together and this sum was divided by the number of litters concerned to secure the coefficient as given in table 3. Likewise the coefficients of variability for all 1st-service litters at thirty days were added together and this sum divided by the number of litters concerned to obtain the coefficient as given in table 3. This method was used on the weights at ninety days to get the coefficient, and a similar procedure used on the weights in the other four service groups to obtain their respective coefficients. For the information of the reader the number of litters concerned in each case is presented in the table. MacDowell ('14, p. 44) shows in studies on weight of adult rabbits that there is less variability within the litters than between individuals of different litters. For this reason and because we wish to compare progeny of different ancestry, the method of expressing the coefficient of variation of the populations as the average of the individual litter coefficients of that population is considered accurate.

Table 3 shows that the coefficient of variation in rabbits is greater at birth than at any other time during our observations. This fact holds good in all service groups. While the coefficient on the average is small, it serves to indicate that prenatal nutrition must be subject to wide variations, otherwise greater uni-

formity in weight at birth should be expected. The thirty-day period is the weaning time for all of the litters studied in this experiment. We note from the table that the coefficient of variation falls below what it was at birth in all service groups. Here again there is no evidence of an increased percentage of 'weak' offspring in advanced service groups, for if such were the case we should expect the coefficient to increase when the animals were thrown into competition for nutrition during the first thirty days of postnatal life, and even one inferior individual would alter the coefficient for the litter. At the ninety-day period there is again a decrease in the coefficient of variation in all service groups, except the 15th- and 20th-service groups. The large size of the probable error here indicates that the 15th- and 20th-service groups cannot safely be assumed to be exceptions.

Taking up a comparison of the coefficients for the different service groups, there appears to be slightly less variability in the offspring as the number of services increases, but this decrease is not universal. Since the probable error is rather large, this difference is no way significant. As has been previously stated, there is also a slight reduction in the number in the litters in the same direction. Our data show us further that there is less variability in the smaller than in the larger litters. This fact affords us an explanation for the slight reduction in the coefficient of variation as the number of services increases.

In table 3 a further fact seems apparent that occasional genetically weak offspring do not occur in any one of the service group more frequently than in any other service group. The table also shows us that for the first ninety days of postnatal growth there is a tendency for individuals of the same litter to approach nearer to a mean weight than was the case either at birth or at thirty days of age. Fetal nutrition is thus more variable than either the nutrition furnished by the mother during the first thirty days after birth or the ordinary food supply furnished from thirty days to ninety days.

TABLE 4

Service-group coefficients of variability at three different periods

| AGE | SERVICE | | | | | | | | | |
|--------------------|-------------------|------------|-------------------|------------|-------------------|------------|-------------------|------------|-------------------|------------|
| | 1st | | 5th | | 10th | | 15th | | 20th | |
| | Number of litters | Per cent | Number of litters | Per cent | Number of litters | Per cent | Number of litters | Per cent | Number of litters | Per cent |
| <i>days</i> | | | | | | | | | | |
| Birth | 26 | 17.62±1.65 | 20 | 28.10±3.10 | 20 | 23.03±2.46 | 13 | 15.59±3.39 | 16 | 21.94±2.62 |
| 30 | 23 | 19.70±1.96 | 18 | 42.65±4.80 | 21 | 35.53±3.69 | 13 | 52.13±6.91 | 11 | 46.37±6.66 |
| 90 | 23 | 24.25±2.41 | 17 | 23.89±2.76 | 21 | 19.63±2.04 | 11 | 24.97±3.53 | 9 | 30.91±4.92 |
| Weighted average.. | | 24.40 | | 31.56 | | 26.11 | | 34.73 | | 31.65 |

3. Service group coefficients of variability

In table 4 are presented the service-group coefficients of variability for all of the progeny studied in the experiment. These coefficients are obtained in the following manner: The sum of the mean individual weights of each litter in the 1st-service group was divided by the number of litters, to get an average at birth, at thirty days, and at ninety days. The standard deviation of this average was then calculated and the coefficient of variability (*e*) obtained by the formula:

$$\frac{\text{Standard deviation of the average}}{\text{Average}} = C$$

The same method was used for all five service groups.

The service-group coefficient of variability differs from the litter coefficient of variability given in table 3 in that the former measures the range in weight between the individual litter means of the different service groups, while the latter is a measure of the range in weight between individuals of the same litter.

The service-group coefficient of variability is valuable in studying the effects of heavy service of males upon the growth in body

weight of their offspring because it will bring to light occasional litters in which every individual is inferior. For example, table 3 shows that there is not an occasional inferior individual in the advanced service progeny. This fact does not remove the possibility of some entire litters being inferior because it is possible to conceive that at one time a male rabbit might sire an exceptionally good litter on the 15th or the 20th service because of extra high reserve, but the majority of his progeny might be inferior in growth as entire litters. By table 4 we shall attempt to discover if litters as a whole are inclined to be more variable in any particular service group.

Table 4 shows that at birth there is less variability in the 1st-service progeny than in any other progeny. This implies that the individual mean weight of the 1st-service litters more nearly represents the mean of every litter in the service group than is the case in any of the other four service groups. There appears to be little tendency for variability to increase as the amount of service increases as shown in the other four service groups at birth.

Concerning the variability between litters at thirty days of age, practically the same relationship exists between the progeny of the different service groups as has been already noted in considering the progeny at birth. The table shows us one additional fact at the thirty-day age; namely, that the greatest variability in weight during the ninety days of the observation exists at weaning time or thirty days. This fact is additional evidence that the nutrition furnished by the mother while suckling the young may vary in absolute amount or may be distributed in limited quantities because of the large number of individuals that she may suckle.

At the age of ninety days there is a striking uniformity in the coefficients for all five service groups. Only in the case of the 20th-service group is there any noticeable digression, and this is probably due to the small number of litters concerned.

Table 4 as a whole does not in any way indicate that inferior litters exist more frequently in any one service group than in any other, and the fact has already been pointed out in connection

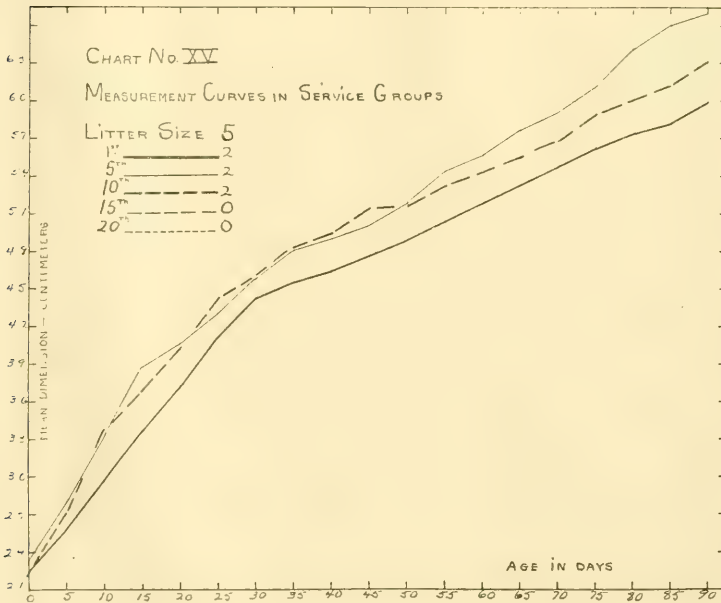
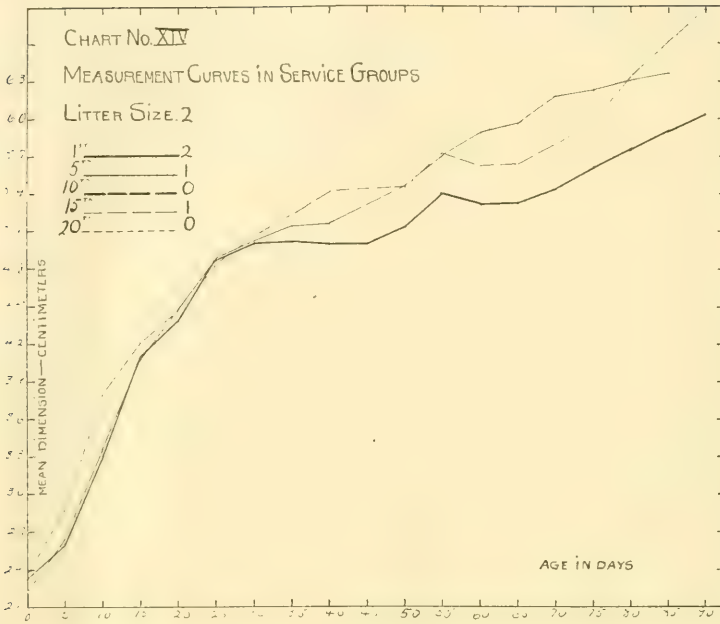
with the study of the weight graphs than in average body weight the advanced service litters are equal and in some cases superior to that of the litters in the light service groups. The fact that variability within litters is small compared with the variability in service groups is well illustrated by a comparison of the coefficients in tables 3 and 4.

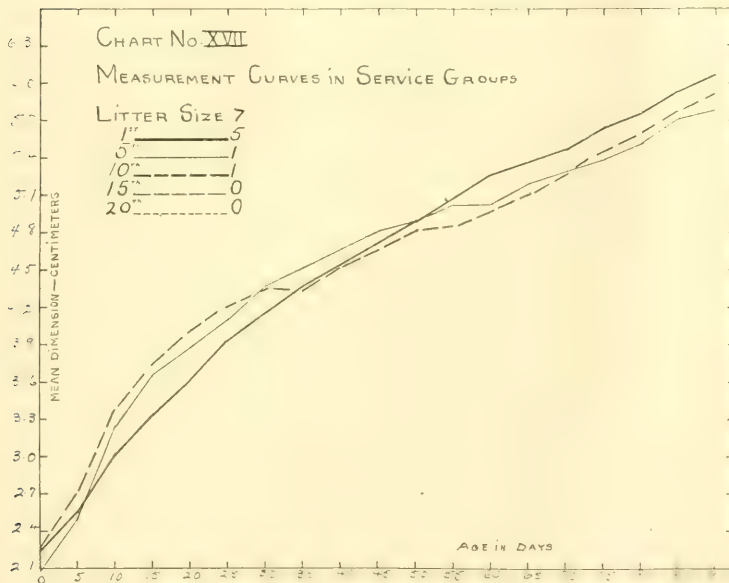
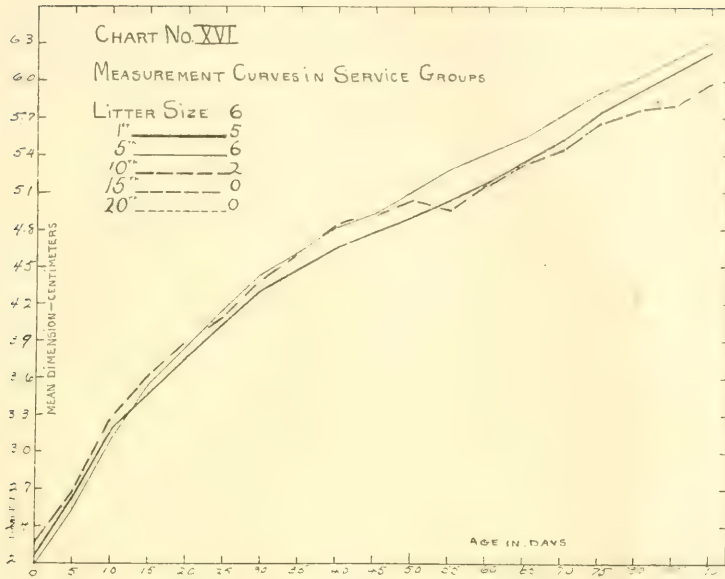
4. Growth by measurements as related to frequency of copulation

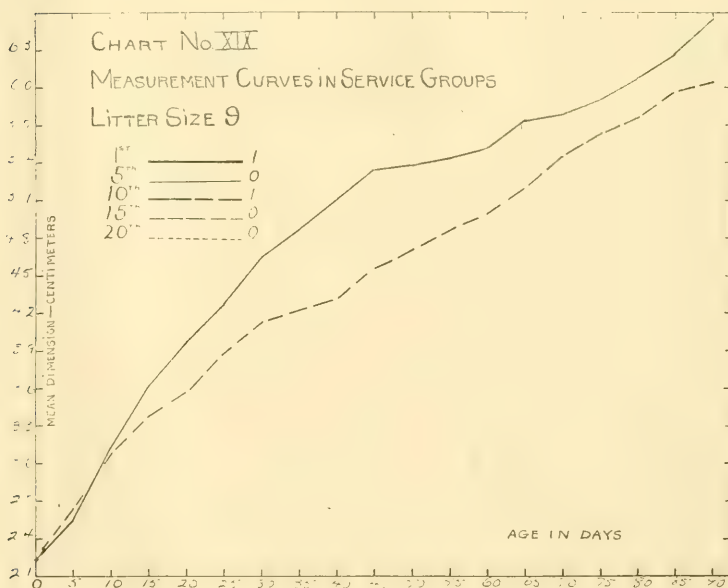
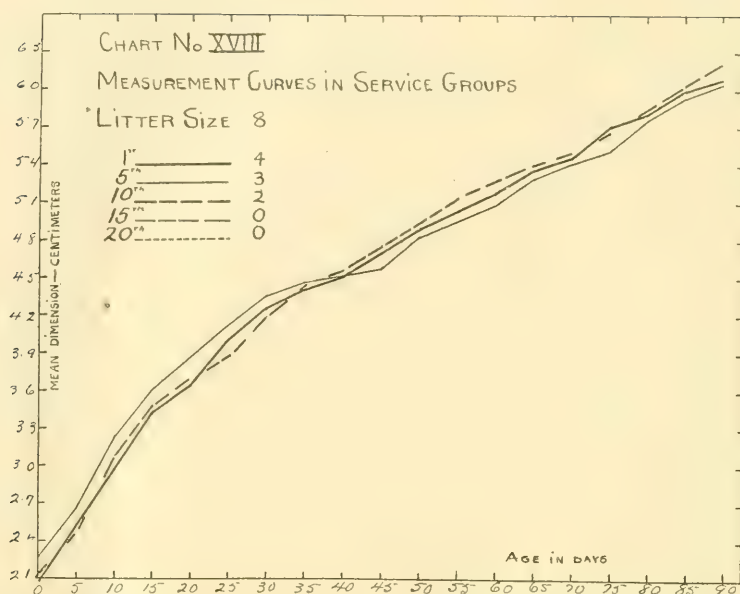
Charts 14 to 21 are presented to show the growth in the mean dimension as obtained on forty-five litters. The method of making measurement and the determination of the mean dimension have been already explained, pp. 581-582. Each graph represents averages of the mean dimension for all litters of the same size in the respective service groups. The mean dimension for a litter is obtained by adding all head measurements to all measurements of ilial extremes and dividing the sum by the total number of readings included in the sum. The expression thus obtained is the average individual mean dimension for the respective litters and may be compared with the average individual weights used in the previous charts.

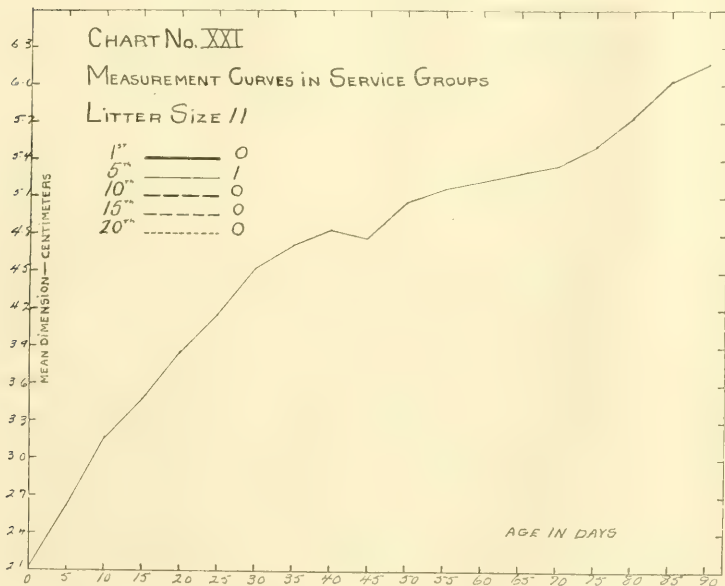
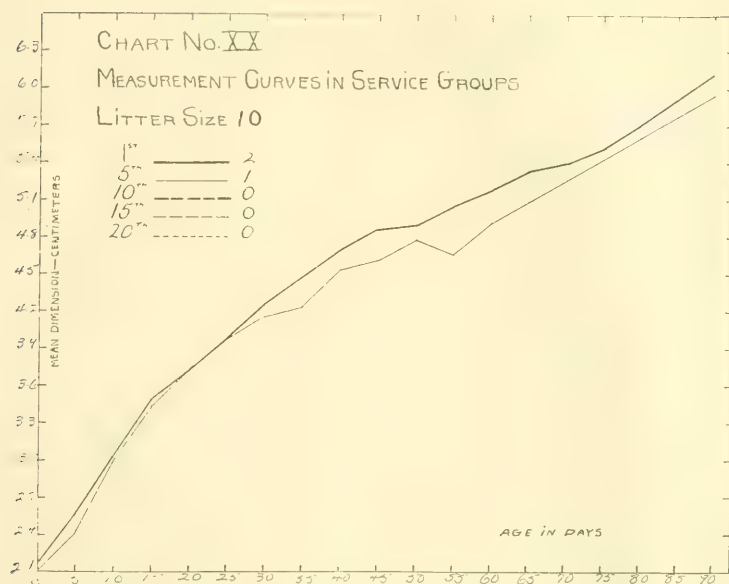
These charts of body development show that there is a maximum increase in the mean dimension up to about the twentieth day, after which there is a very noticeable flattening of the graphs. From about the twentieth day on to the end of the observations at ninety days the progressive increase in the mean dimension is about constant. The increase in the mean dimension is thus in marked contrast to the increase in body weight previously illustrated by charts 1 to 13. Body weight has been shown to make a rather constant increase up to the end of ninety days, and this is well illustrated by the fact that the weight graphs show little if any tendency to flatten out.

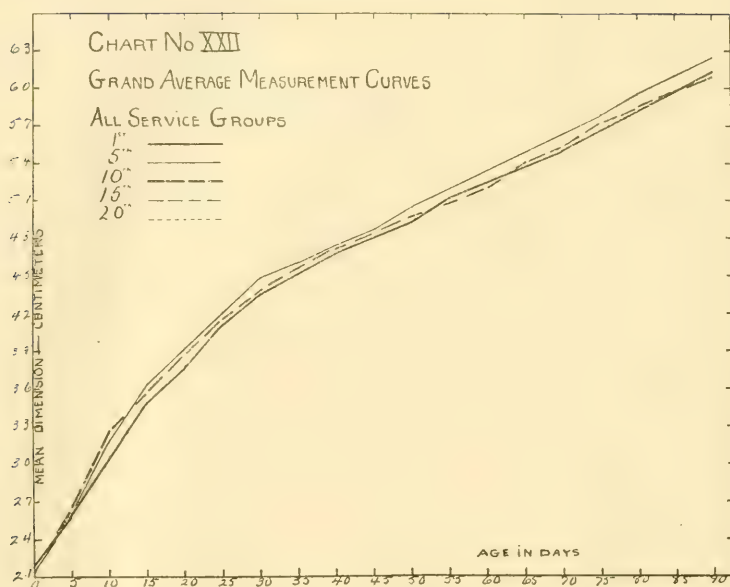
Though the number of litters making up a mean dimension graph is in most cases small, they serve to illustrate the same principle as the weight graphs, namely, that the advanced service progeny are fully equal to the 1st- or 5th-service progeny at all times during the ninety days of the observation. On











charts where but a single litter makes up a graph a rather sudden break may sometimes be noted in the graph. This, in our opinion, is the result of error in measurement, and for this reason the graphs made up of several litters will be less influenced by minor errors and hence should be more representative of actual dimensions.

In chart 22 are presented grand average graphs made up as the average of twenty-one 1st-service litters, fifteen 5th-service litters, eight 10th-service litters, and one 15th-service litter. Here the coincidence of the 1st-, 5th-, and 10th-service graphs is very striking. This fact bears out our previous conclusions from body weight studies that heavy sexual service of the male has no effect upon the growth of his offspring. Our evidence in studying the increase in the mean dimension does not show any effect on the progeny, from the heavy service of the male. The 15th-service graph is made up of but one litter of two individuals sired by Male No. 1 and out of an average sized female. The fact that this litter is few in numbers and has as a sire the largest of the males will probably account for their larger mean dimension.

TABLE 5

Percentage mortality in offspring during the first five days of life and between the fifth and the ninetieth day of life

| | SERVICE | | | | |
|---|---------|-------|-------|-------|-------|
| | 1st | 5th | 10th | 15th | 20th |
| Number of animals born..... | 180 | 119 | 139 | 84 | 77 |
| Number dying first five days..... | 16 | 15 | 16 | 11 | 7 |
| Per cent dying first five days..... | 8.89 | 12.61 | 11.51 | 13.09 | 9.09 |
| Number dying between 5 and 90 days..... | 21 | 36 | 17 | 9 | 19 |
| Per cent dying between 5 and 90 days..... | 11.67 | 30.25 | 12.23 | 10.71 | 24.68 |

Summarizing the results of the measurement studies, we note that there is very close proximity of the graphs for the different service groups. This points very strikingly to the probable fact that heavy service of males has no effect upon the growth of their offspring in the length of head and in the breadth of ilial expanse.

In table 5 the progeny are grouped by services and the number and the percentage mortality is given for each service group. Under the row marked "Number dying first five days" are included all animals dead at birth as well as those that died during the first five days of life. The other row of the table includes only animals actually dying between the fifth and the ninetieth day of postnatal life.

The percentage of mortality during the first five days shows a slight increase as the number of services increases up to the 15th-service group. Comparing the 1st-service group with the 20th-service group, we note that the percentage mortality in the first five days is practically the same in both groups. Since the environment has been more favorable for the 20th-service litters than for the 1st-service litters, as previously pointed out, there is no indication that twenty copulations by a male do in any way tend to reduce the percentage of his progeny that will survive the first five days of postnatal life. The table shows practically the same percentage of mortality during the first five days in both the 5th- and the 15th-service groups. The explanation for the rather high percentage of mortality in the 5th-service group is that two litters were destroyed outright by the mother and a

number of the other 5th-service litters were born during extremely hot weather when the mortality was very high even among the older animals. The 10th-service group shows a higher death rate than the 1st-service group. In all these cases the percentage of mortality during the first five days does not seem to depend upon the number of services that the male is required to make.

Table 5 shows that there is very little consistency between the mortality percentages as revealed in the first part of the table and between the percentages of deaths that occurred between five and ninety days. The first five days is a very critical time in the life of the young rabbit and very slight exposure may bring disaster. When this period is over the deaths usually result from bowel disorders or from septicaemia. Bowel disorders are most common during the very hot weather of summer in the stock, and it is very unfortunate that a large number of the animals in the 5th-service groups should have been so attacked. The 10th-service progeny also show a higher death rate than the 1st-service progeny, even though these 10th-service litters were housed under more favorable conditions than were the majority of the 1st-service litters. The mortality percentage of the 15th-service offspring is the highest of any of the service groups during the first five days of life, but it falls below that of all other service groups between the age of five and ninety days. Practically one-fourth of the 20th-service rabbits died between the fifth and the ninetieth day of postnatal life. An outbreak of septicaemia happened to occur among a number of these litters. This being the case, we are inclined to believe that this sudden outbreak of disease rather than any inherent weakness of the progeny resulting from heavy sexual service of the sire is here operating to cause the high percentage of mortalities.

6. Relation of number of services made to sex of offspring.

A study of the relation of sex of the offspring to the amount of sexual service the male is required to perform is important because such data will show if either male or female producing

TABLE 6
Sex ratios in service groups. Males to 100 females

| | SERVICE | | | | |
|--------------------------------------|---------|-----|------|------|------|
| | 1st | 5th | 10th | 15th | 20th |
| Number of individuals concerned..... | 78 | 76 | 117 | 84 | 77 |
| Ratio..... | 129 | 77 | 80 | 53 | 28 |

sperm (Bachhuber, '16) is weakened by excessive functioning of the male reproductive organs. Table 6 presented below shows the sex ratio of the offspring in the different service groups.

Table 6 shows that in the 1st-service group there are 129 males to every 100 females. After the 1st-service group there is a regular decline in the number of males produced, with the exception of the 10th-service group. There is apparently some underlying cause to bring about the high percentage of females to males in all the advanced service groups, and there is a direct relation between the amount of service previously performed by the male and the proportion of female offspring that he will sire.

The properties of the spermatozoa are perceptibly modified by heavy sexual service of males (Lloyd-Jones and Hays, '17), there being a larger percentage of weak sperm in the advanced service sperm.

Two possibilities exist: either female-producing spermatozoa are formed more largely than male-producing spermatozoa as the amount of service of the male increases or the male-producing sperm are in themselves weaker than the female-producing sperm and consequently fewer of them survive to take part in fertilization. On the first point there is no evidence available. Concerning the second point, Stockard ('13) offers the hypothesis that in the case of guinea-pigs the larger female-producing sperm are more affected by alcoholization of the male than the smaller male-sperm producing. In the case of excessive sexual service, however, the large female sperm may be more vigorous because of their size or their greater chromatin content and thus out-distance the male-producing sperm in the struggle of fertilization, thus giving a higher percentage of female progeny in the heavy service groups as compared with the light service groups.

TABLE 7

Sex as related to mortality. Percentage mortality of the sexes

| PERIOD | SEX | SERVICE | | | |
|-----------------------------------|-----|---------|-------|-------|-------|
| | | 5th | 10th | 15th | 20th |
| First five days..... | ♂ | 8.69 | 7.14 | 0 | 9.09 |
| | ♀ | 6.85 | 11.90 | 17.74 | 9.09 |
| Between five and ninety days..... | ♂ | 6.52 | 8.93 | 9.09 | 27.27 |
| | ♀ | 12.33 | 15.47 | 11.29 | 24.24 |

In table 6 we considered the relation of sexual service to the sex of the offspring and found that a predominance of females to males is the rule in the heavy service groups. In table 7 we shall consider sex of the offspring dying before the close of the observation period at ninety days.

Table 7 shows that up to the 15th-service group there is a higher death rate among the female offspring than among the male offspring. In the 20th-service group, however, the fact will be noted that females are just as likely to survive as males for the first five days of postnatal life. Between the fifth and the ninetieth day there is a slightly lower death rate of females than males in the 20th-service group. These facts seem to indicate that in comparison with males of the same class, female offspring from the 20th-service are in respect of their ability to survive superior to ordinary offspring from the less advanced service groups. The fact still seems evident that these female offspring in the 20th-service group are slightly more likely to die than ordinary offspring.

SUMMARY OF FACTS

1. Body weight of the rabbit is a measure of growth that is subject to considerable variations largely brought about by slight changes in the environment.

2. The rate of increase in body weight continues at a uniformly rapid rate for the first ninety days of the rabbit's life.

3. The factors that appear to govern the weight of the young at birth are age of mother, state of health of mother, weight of

mother, weight of sire, character of food of mother, and number of individuals born in the litter.

4. The factors that govern the rate of postnatal growth of the young for the first ninety days are weight at birth, number in litter, milk supply furnished by the mother, and, after weaning, the character of the food supplied to the young and general character of the quarters.

5. No inferiority in the offspring from the heavy service groups is revealed by comparing the body weights with those of the light service groups.

6. The average litter coefficient of variability in body weight at birth at thirty days and at ninety days is no greater in the progeny in the heavy service groups than in the light service groups. Greater variability might be expected if a part of the offspring are made genetically inferior by inferiority of the male element in the advanced service groups.

7. The service group coefficients of variability indicate greater variability in the weight of the general population than within the litters, but do not indicate that heavy service produces 'weak' litters.

8. Body development seems to progress at the maximum rate during the first twenty days of postnatal life, after which time there is a rather marked decline in the rate of increase in head length and breadth of ilial expanse.

9. No inferiority in the offspring from the advanced services is revealed from a study of body growth by measurement.

10. Offspring in the more advanced service groups do not show a significantly higher percentage of mortality during the first five days of life than do the offspring in the light service groups.

11. A higher mortality does not seem to exist in offspring from the advanced service groups as compared with the light service groups between the ages of five and ninety days.

12. Heavy sexual service of males gives a decrease in the proportion of male to female offspring that is very perceptible.

13. Female offspring are to some degree more likely to succumb than male offspring in all service groups except the twentieth.

14. The high percentage of deaths of female progeny is largely due to the predominance of females to males in the litters.

15. By no means thus far used has any inferiority of progeny from heavy sexual service been discovered. They are fully equal if not superior to progeny from very light service of male.

DISCUSSION

The amount of sexual service that the male performs has a marked effect upon the physical properties of his spermatozoa (Lloyd-Jones and Hays, '17); the whole basis of this work is to discover if these effects are in any way made manifest in the offspring.

Growth in body weight must be assumed to be due to a complex of stimuli acting upon every living cell of the organism. If it were possible to modify the contribution of growth stimuli from the male germ cell by extreme sexual use of the male, an effect should be produced upon every cell of the body in his offspring and a reduction of these stimuli would thus result in a decreased body growth. The sum total of the body increase in the offspring from the heavy service series is fully equal and even superior to the increase in the offspring in the light service groups. This apparent superiority has been attributed to various factors, largely environmental and possibly to superior male reproductive cell. After these factors are corrected for, which we have found impossible to do, we believe that the rate of growth in body weight would be identical in all five service groups. A study of body weight as reported here will only reveal the character of the total population and will not reveal the occurrence of an occasional inferior individual.

The coefficient of variability of litters, on the other hand, is valuable in that it will reveal the occasional inferior individual in the litter. If only a part of the offspring in the heavy service groups are inferior as far as rate of growth is concerned, there should be a greater coefficient of variability in the litters from heavy service than among the light service litters. No such evidence appears in our data, and this fact we feel warrants the assumption that not even a part of the offspring in the

heavy service group are more inferior as far as ability to increase in body weight is concerned than the offspring in the light service groups.

The service group coefficient of variability does not reveal that any inferiority of entire litters is brought about by heavy sexual service of males. This coefficient does show that the largest coefficient of the first ninety days of postnatal life is found just at the close of the suckling period at thirty days. The coefficient further shows that the variability in weight of the general population is much greater than within the litters.

Body measurements furnish us with further material for the study of the offspring in the different service groups. These data do not reveal any new facts to indicate any greater inferiority of offspring in any one of the five service groups. Here again the same modifying factors have been in operation that have affected the body-weight data, and a correction, if possible, for these we think would show that the offspring in all five of the service groups are identical in body dimensions.

Concerning the question of rate of mortality in progeny from light and heavy service, we have no evidence that there is a higher death rate in the advanced service groups over that observed in the light service groups.

A direct relation apparently exists between the amount of sexual service of males and the percentage of females that they will sire. The ratio of males to females is highest in the 1st-service group and progressively decreases up to the 20th-service group. There is a possibility that heavy service exerts a selective action upon the sperm cells and may eliminate from fertilization the majority of the male-producing spermatozoa. The large female-producing sperm cells may show a greater rate of motility, greater endurance, or for some other cause out-distance the male-producing spermatozoa, thus resulting in a preponderance of female offspring in advanced service groups.

A possible explanation for the high percentage of deaths among females lies in evidence showing that the percentage of female offspring is increased by heavy service of the male as shown on page 607. The weight (Minot, Jackson, King) of

female offspring in multiparous animals at birth is slightly less than that of the males. If this is true for the rabbit, it may render the females less able to compete with the male offspring for nourishment during their early life when food supply is of such vital importance in determining the survival of the young. The fact that the great majority of the offspring dying in early life have been females seems to warrant the assumption that females are actually less able to compete with the males during the early part of life. The data do not justify the conclusion that there is any higher rate of mortality in the advanced service groups than in the lighter service groups after the first five days of postnatal life. If inferiority of offspring exists in the advanced service groups because of the predominance of females, which we may assume under all ordinary conditions are less able to survive than males, it is apparent that no real inferiority exists, but that the mortality is greater because the percentage of females is greater in the heavy service groups.

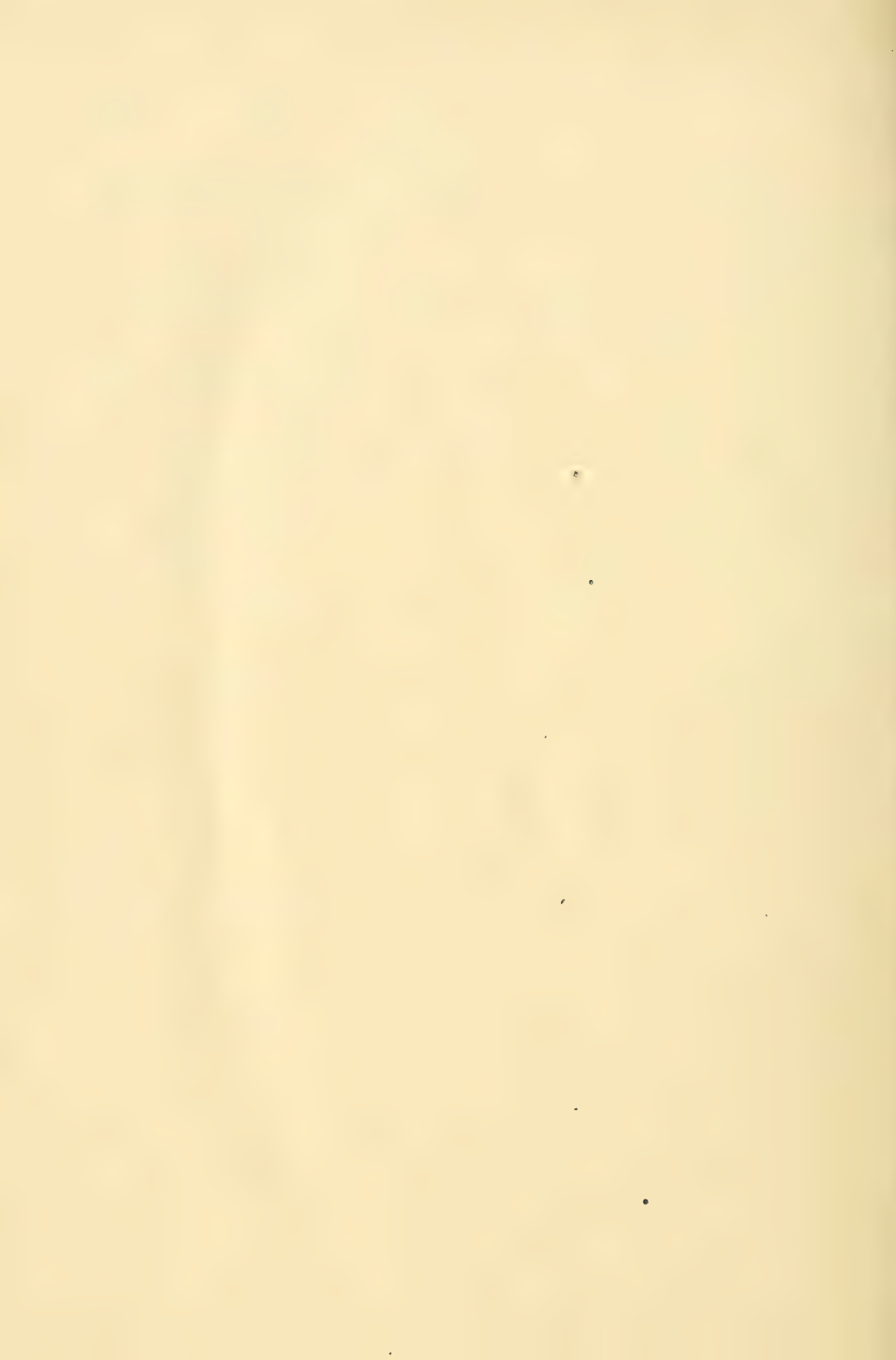
In conclusion, it may be noted 1) that the methods used for measuring the character of offspring from different degrees of sexual service of sires fail to show that any inferiority of the offspring can be induced by using a male excessively; 2) that the male in heavy sexual service furnishes germ cells that are fully the equal in their contribution to his offspring of those elaborated by a male in very moderate sexual service.

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